# BEHAVIOR AND WELFARE OF THE INDIVIDUAL WITHIN LARGE, COMMERCIALLY-RELEVANT GROUPS

EDITED BY: Michael Toscano, Dana L. M. Campbell and Rebecca K. Meagher
PUBLISHED IN: Frontiers in Veterinary Science

frontiers Research Topics

# frontiers



ISSN 1664-8714
ISBN 978-2-88966-775-8
DOI 10.3389/978-2-88966-775-8

## About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

## Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

## Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

## What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

# BEHAVIOR AND WELFARE OF THE INDIVIDUAL WITHIN LARGE, COMMERCIALLY-RELEVANT GROUPS

Topic Editors:
**Michael Toscano,** University of Bern, Switzerland
**Dana L. M. Campbell,** Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia
**Rebecca K. Meagher,** Dalhousie University, Canada

**Citation:** Toscano, M., Campbell, D. L. M., Meagher, R. K., eds. (2021). Behavior and Welfare of the Individual Within Large, Commercially-Relevant Groups. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-775-8

# Table of Contents

frontiers in Veterinary Science

EDITORIAL
published: 23 March 2021
doi: 10.3389/fvets.2021.656236

# Editorial: Behavior and Welfare of the Individual Within Large, Commercially-Relevant Groups

*Michael J. Toscano*[1]*, *Rebecca K. Meagher*[2] *and Dana L. M. Campbell*[3]

[1] Center for Proper Housing: Poultry and Rabbits (ZTHZ), Division of Animal Welfare VPH Institute, University of Bern, Bern, Switzerland, [2] Dalhousie University, Halifax, NS, Canada, [3] Agriculture and Food, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Armidale, NSW, Australia

**Keywords: welfare, tracking, precision livestock farming, livestock, agriculture, data analytics**

## Editorial on the Research Topic

## Behavior and Welfare of the Individual Within Large, Commercially-Relevant Groups

Individuality is increasingly recognized as important and has become a focus of exploration in many fields that study behavior and other responses. Specifically in Animal Welfare where a variety of responses (e.g., behavioral, endocrinological, immunological, neurological, etc.) must be amalgamated comprehensively as animals adapt to stressors, animal individuality should be considered essential. The shift toward a focus on individuals (from group-level observations) has largely been driven by a growing awareness that differences between individuals can be consistent over time and across varied settings (1–4). With the increased attention toward individuality and its incorporation into our understanding of Animal Welfare, we should be encouraging and utilizing the most modern and advanced methods at our disposal. The current Research Topic comprises 10 articles that utilize novel methods to assess animal behavior, often using advanced sensor technologies and analytics, and answer questions about its relationship to welfare and physiological functioning.

Measuring the behavior of individuals in their current housing environments should consider long-lasting impacts of previous environments. Campbell et al. looked at the impacts of different types of rearing enrichments on subsequent ranging behavior in free-range hens. Radio-frequency identification technology was used to track individuals across a flock cycle to show that the type of environment they were reared in, affected how much they utilized the range area as adults with rearing perching structures increasing range usage.

The impacts of rearing environments as well as subsequent individual variation in ranging behavior on hen welfare was further explored by Bari et al.. Regular external measures of bird welfare such as body weight, plumage coverage, and comb wounds showed that hen welfare was affected by their rearing environment with control-reared hens showing worse feather coverage with age. There was also a relationship with range use where hens that spent more time outside had better feather coverage.

Developmental impacts on behavior can even precede the rearing environment, occurring via maternal stress and hormone deposition in chicken eggs. Peixoto et al. assessed offspring of stressed hens for anxiety-like and fear responses. The behavioral tests demonstrated limited impacts of maternal stressors and impacts did not appear to be caused by corticosterone depositions. The greatest differences were found among varying genotypes demonstrating the importance of considering the overarching influence of animal strains on individual variation in behavior when drawing general conclusions based on a specific breed.



***Edited and reviewed by:***
*Jeremy N. Marchant, Livestock Behavior Research Unit (USDA-ARS), United States*

***Correspondence:**
*Michael J. Toscano*
*michael.toscano@vetsuisse.unibe.ch*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *20 January 2021*
***Accepted:*** *26 February 2021*
***Published:*** *23 March 2021*

***Citation:***
*Toscano MJ, Meagher RK and Campbell DLM (2021) Editorial: Behavior and Welfare of the Individual Within Large, Commercially-Relevant Groups. Front. Vet. Sci. 8:656236. doi: 10.3389/fvets.2021.656236*

While a focus on the individual is critical for direct understanding of behavior and welfare impacts, livestock animals are typically housed in groups and will thus be affected by those individuals surrounding them. Keshavarzi et al. analyzed GPS data to demonstrate that naïve cattle in paddocks with a virtual fence were facilitated by their herd mates when learning the correct responses to this new technology that signals the presence of an invisible fence line via audio and electrical cues.

Advances in sensor technology and computational abilities often require novel statistical methods to ensure generated data is used most effectively. In an example of how large complex datasets can be analyzed using these novel methods, McVey et al. examined the sequential order of dairy cows entering the milking parlor over a 6-month period using entropy as a tool to assess variance and the relationship to social and temporal correlates. Interestingly, cows at the front and rear of the queue proved to be most consistent, a feature reminiscent of classical concepts of dominance hierarchies. Further analysis using machine learning proved effective in relating the variables with productivity, health status, and behavior within the home pen.

Similarly, Chopra et al. examined the structural consistency of a commercial dairy herd using remote identification of pairs of cows within 3 meters of each other for more than 60 s. Proximity networks were then related to feeding-specific areas and the entire barn, as well as health status and productivity factors. The study employed applied network visualization and social network analysis to determine that associations of dyads were non-normal but rather related temporally and to specific barn areas.

Baur et al. described the development of a radiography protocol for assessing keel bone health in conscious laying hens. The radiographs distinguished between different types of fracture. They demonstrated that keel bone fractures are likely more common than was previously recognized, with 97% of the 150 birds studied having at least one fracture, showing that this method, which is easier to use on farm than other scanning technology, provides more detail and likely greater sensitivity than the common approach of physical palpation.

Bone health in poultry is an example of how health outcomes can be linked with individual developmental and psychosocial factors. Rokavec and Šemrov thus hypothesized that low body weight and high fear or stress would associate with bone condition. Although they did not find that low body weight or indicators of fearfulness and corticosterone levels in the feathers of younger birds predisposed them to bone deviations or fractures, such bone damage during lay was associated with lower concurrent open field activity and higher later sociality, posing questions about the causal relationships between putative indicators of fear, stress, social behavior and physical health in this species.

van der Zande et al. meanwhile, tested new behavioral analyses to quantify what they considered Dynamic Indicators of Resilience in pigs: measures of consistency in physical activity levels, automatically detected by ear tag accelerometers on piglets injected with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). They found that changes in skewness from pre-to post-injection predicted mortality risk, and that statistical tendencies suggested possible relationships between high variation in activity, as measured by the Root Mean Square Error (RMSE) in the days after the injection and its increase from pre-exposure levels, and clinical illness symptoms. This novel use of accelerometer data thus holds some promise for quantifying individuals' resilience to health challenges.

Finally, individual monitoring of behavior can provide insight into changes in the brain, as demonstrated by Armstrong et al. who tracked over 400 hens using RFID technology and correlated this with responses to standardized behavioral tests and brain samples from a subset of the hens. The results showed that increased ranging behavior may stimulate cell proliferation, a measure of plasticity, in the rostral hippocampus, thus contributing to the cognitive benefits of outdoor access, and that overall proliferation correlated with a personality indicator, tonic immobility as a fear response.

Overall, this body of research highlights the use of technology for precise data collection at the level of the individual and novel analysis methods to better understand factors that affect animal behavior and welfare as well as how these individuals are influenced by the groups they are housed within.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## REFERENCES

1. Svendsen GE, Armitage KB. Mirror-image stimulation applied to field behavioral studies. *Ecology*. (1973) 54:623–27.
2. Gosling SD. From mice to men: what can we learn about personality from animal research? *Psychol Bull*. (2001) 127:45–86. doi: 10.1037/0033-2909.127.1.45
3. Réale D, Dingemanse NJ, Kazem AJN, Wright J. Evolutionary and ecological approaches to the study of personality. Phil Trans R Soc B. (2010) 365:3937–46. doi: 10.1098/rstb.2010.0222
4. Mittelbach GG, Ballew NG, Kjelvik MK. Fish behavioral types and their ecological consequences. *Can J Fish Aquat Sci*. (2014) 71:927–44. doi: 10.1139/cjfas-2013-0558

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

METHODS
published: 12 March 2020
doi: 10.3389/fvets.2020.00129

# Radiographic Evaluation of Keel Bone Damage in Laying Hens—Morphologic and Temporal Observations in a Longitudinal Study

*Sarah Baur*[1], *Christina Rufener*[2†], *Michael J. Toscano*[2] *and Urs Geissbühler*[1*]

[1] Clinical Radiology, Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Bern, Bern, Switzerland, [2] Center for Proper Housing: Poultry and Rabbits, Animal Welfare Division, Veterinary Public Health Institute, Vetsuisse Faculty, University of Bern, Bern, Switzerland

OPEN ACCESS

**Edited by:**
Stephanie Torrey,
University of Guelph, Canada

**Reviewed by:**
Carla Molento,
Federal University of Paraná, Brazil
Sezen Özkan,
Ege University, Turkey

***Correspondence:**
Urs Geissbühler
urs.geissbuehler@vetsuisse.unibe.ch

**†Present address:**
Christina Rufener,
Department of Animal Science, Center for Animal Welfare, University of California, Davis, Davis, CA, United States

**Specialty section:**
This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science

**Received:** 07 November 2019
**Accepted:** 20 February 2020
**Published:** 12 March 2020

**Citation:**
Baur S, Rufener C, Toscano MJ and Geissbühler U (2020) Radiographic Evaluation of Keel Bone Damage in Laying Hens—Morphologic and Temporal Observations in a Longitudinal Study. Front. Vet. Sci. 7:129. doi: 10.3389/fvets.2020.00129

The keel bone of commercially kept laying hens is known to be frequently affected by morphologic changes such as fractures and deformations with important implications for animal welfare. To detect morphologic changes, various methods such as palpation, computed tomography, and ultrasound are available, though radiography allows for the greatest level of detail in combination with the most ease of use. To explore the benefits of radiography in providing objective data on keel fractures from the age of 22–61 weeks within a single laying period, the keel bones of 75 Lohmann Brown and 75 Lohmann Selected Leghorns were radiographed every 3 to 5 weeks. Type, location, angulation, dislocation, callus formation, and healing process were assessed descriptively for each lesion. Ninety-nine percent of the animals showed at least one keel bone lesion during the study and 97% of the animals had at least one keel bone fracture. In 77% of the cases, the caudal third of the keel bone was affected. The fracture types were transverse and oblique (88%), comminuted, and butterfly. Further lesions were sclerosis, new bone formation and angulation. For each keel bone, an average of three fractures (3.09 ± 1.80) was detected at the end of the study. The described radiographic protocol for keel bone lesions was suitable for longitudinal, on-site examinations in conscious laying hens. Our results also indicate that keel bone fractures are more frequent than reported in earlier studies. The described radiographic examination protocol can be used to perform comparative studies of palpatory findings, or to assess the clinical significance of different fracture types which require a high level of detail.

**Keywords: fracture, aviary system, imaging, age, x-ray**

## INTRODUCTION

Housing of laying hens for egg production is known to be associated with skeletal problems such as fractures and deformities of the keel bone (1). The causes of such fractures have not been definitively identified but are suspected to be a multifactorial problem including: genetic regulation of bone health and high egg laying performance (2, 3), bone calcium depletion, and collisions within the housing systems (4–6).

The most common method for evaluating whether laying hens have keel bone fractures (KBF) is palpation, a relatively simple and low-cost method that allows for longitudinal observations. Despite these benefits, palpation requires assessors to undergo training and evaluation to ensure reliable and accurate results (4, 7). Even with superior training, it is likely that a large percentage of fractures will be missed due to a variety of reasons including: fissures, inability to detect fractures on the dorsal aspect of the keel, or damage hidden by the large breast muscle group.

Due to these concerns, alternative techniques should be evaluated that would allow for reliable, longitudinal assessment of KBF. Radiography, a well-established method for fracture detection (8), has been used to detect KBF in several, non-commercial (9–11) as well as quasi-commercial (12) settings.

An evaluation protocol was developed for scoring gross severity of fractures (available at: http://www.keelbonedamage.eu/activities/practical-information-for-stakeholders/online-tool-for-evaluating-fractures-from-radiographic-images/>) that was determined to be reliable in terms of intra- and inter-observer reliability (12). The protocol was successfully used to grade fractures in relation to hen productivity (12) and mobility (13). Although Rufener et al. (12) included presence of a fracture gap to indicate healing, the protocol was fairly narrow in scope. For instance, the protocol did not classify the location, type, or size of the fracture. Given the variation of these fracture features in commercial laying hens, it is likely that damage will have variable effects on hen welfare and productivity where detailed information about fracture characteristics might aid disentangling aspects of clinical significance. For instance, it is unknown if small fractures at the caudal tip are associated with pain differently than fractures similar in size but located on the cranial aspect of the keel. In the same vein, different types of fractures may be associated with different causes, e.g., external forces resulting in traumatic injuries vs. pathologic causes due to reduced bone strength (1). Without an objective classification of KBF, efforts at linking the causes and effects of different types of fractures are severely hindered.

The aim of this study was to describe a radiographically based, objective, fracture-specific characterization of KBF. Our methodology specifically is intended for longitudinal observations and considers changes in individual fractures over time, specifically a 40-week interval within a single laying period.

## MATERIALS AND METHODS

The study was performed in a barn managed by the Aviforum (www.aviforum.ch), a contract research facility that owned the animals and was the sole provider of animal care. All animals were sourced from a commercial hatchery and then slaughtered at an abattoir per common industry practice at the conclusion of the laying period. Under a long-standing agreement between the Aviforum and the Center for Proper Housing of Poultry and Rabbits, 10 individual compartments were used for the study. In each of the 10 compartments, 15 animals from one genetic line [i.e., 5 compartments with 15 Lohmann Brown (LB) animals and 5 compartments with 15 Lohmann Selected Leghorns (LSL) animals] were maintained alongside 210 laying hens of the other hybrid. All the animals were of the same age. Full detail of the barn configuration and management protocols, including rearing, are provided by Rufener et al. (14). A total number of 75 LB and 75 LSL, each of them individually marked with a number at the phalanges, were included in the longitudinal study. All the hens were kept under the same conditions in a Bolegg Terrace aviary system.

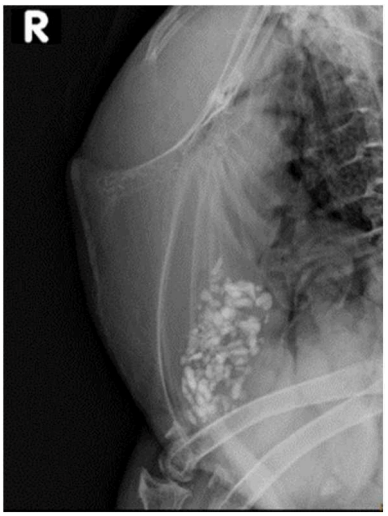


**FIGURE 1 |** Laterolateral view of the keel bone of the free-range laying 10 years old Appenzeller Spitzhaubenhuhn "Esmeralda" without visible fractures or lesions. Limited interpretation of the caudal keel bone tip due to superimposition of one stifle.

The animals were examined over a period of 10 months during which each animal was assessed 11 times (22, 25, 28, 33, 37, 40, 45, 49, 54, 57, and 61 weeks of age). For better understanding, the term "observation Phase (P)" from 1 to 11 is used hereafter. On the examination day, focus animals were caught, placed in transport boxes, and moved to the radiography site within the barn (i.e., within the hygiene barrier), a distance varying between 10 and 30 m depending on the location of the pen. The hens remained conscious and were not anesthetized for the entire procedure.

Based on pilot trials (10), the following slightly adapted radiographic protocol was used. A single laterolateral radiograph of the keel bone (**Figure 1**) was performed with a portable X-ray machine consisting of a focal spot of 1.2 × 1.2 mm (Gierth X-Ray international GmbH), and 46 kV, 2.4 mAs and a focus-film-distance of 80 cm were set. The imaging system consisted of a flat panel detector (CXDI-50G, Canon). The conscious hens were positioned as described by Sirovnic et al. (10). As the size of each hen within a hybrid was consistent, the exposure field had to be readjusted only between the different hybrids. After exposure, the animal was immediately returned back to the crate where it remained until the procedure was completed for all animals, whereupon hens were returned to their home pen. The entire

**Abbreviations:** LB, Lohmann Brown; LSL, Lohmann Selected Leghorns; KBF, Keel bone fracture; P, Observation Phase; SD, Standard deviation.

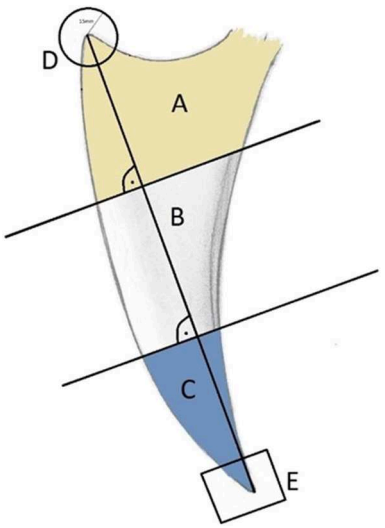


**FIGURE 2 |** Laterolateral view of a schematic representation of a keel bone. The keel bone is divided in three parts, each of them measures one third of the base to apex line: Cranial third **(A)**, middle **(B),** and caudal third **(C)**. The apices are labeled **(D)** (cranial; $r = 15$ mm) and **(E)** (caudal 1/5 of **C**).

process, including collection of hens, imaging, and returning the animals, took approximately 120 min for 75 hens. A radiation protection permission was granted by the Swiss authority for this study (approval number BE-03222.41.013). Approval for use of experimental animals was obtained from the Veterinary Office of the Canton of Bern in Switzerland (approval number BE31/15). The experiment complied with Swiss regulations regarding the treatment of experimental animals.

The images were imported into the Picture Archive and Communication System (IMPAX EE, Agfa Healthcare) of the Vetsuisse Faculty of Berne and evaluated on a radiographic workstation with a certified medical screen (EIZO) with a DICOM radiology evaluation software (IMPAX EE Client, Agfa Healthcare). All evaluations were conducted by a single person (SB) with guidance as needed (UG). The morphology, location, time of first appearance and the change over time of each lesion were reported. Additionally, the soft tissues around the keel bone were evaluated.

The keel bone was initially divided into thirds: A (cranial), B (middle), and C (caudal). In addition, the cranial aspect was denoted by "D" (circle with a radius of 15 mm and a center at the cranioventral tip of the keel bone) and the caudal aspect denoted by "E" (caudal fifth of the caudal third C; **Figure 2**). Fractures and other lesions in "E" were attributed to "C" if not specified. Fractures and other lesions in "D" were attributed to "A" if not specified. Furthermore, it was noted if a lesion involved the dorsal, ventral, cranial or caudal bone surface.

A bone lesion could be either fracture or not fracture-related. A lesion was defined as a fracture if a step at the bone surface or a fracture gap was present. The fractures were divided into categories: transverse, oblique, butterfly, and comminuted. Non-fracture related lesions were sclerosis, new bone formation without a fracture gap, and angulation (**Figure 3**). A transverse fracture was noted, if the fracture line did not deviate more than 10 degrees from the perpendicular line to the base to apex line of the keel bone. If the angle measured 11 or more degrees, an oblique fracture was noted. A butterfly fracture was noted if three main fragments were present and the middle main fragment was roughly triangular. All other fractures with more than two fragments were reported as comminuted fractures. Sclerosis was reported if a bone opacification was detected. A new bone formation was noted in case of superficial new bone formation without the presence of a fracture gap. An angulation was noted in case of a change of the axis within the keel bone.

A healing of a fracture was noted when the fracture gap was no longer visible in subsequent radiographs. It could be accompanied by callus formation. A fracture was also considered to be healed if no gap or superimposition was visible at the time of the first onset, even if a step at the bone surface was present. In addition, the length of each keel bone from the caudal to the cranial tip was measured.

The present study was of an explanatory nature, with the aim to characterize and describe KBF and their change over time, as well as develop a standardized methodology to accomplish said aim. Given these objectives, our methods employed primarily descriptive statistics to describe the temporal occurrence of lesions and fractures, their characteristics (e.g., localization, type, callus formation, soft tissue swelling), or the proportion of hens being affected by a certain type of lesion/fracture. In addition, we grouped collected data at the hen-level to evaluate whether fracture development differed over time between LB and LSL hens using a linear mixed effect model [package "lme4" (15)] in R 3.4.2 (16). The outcome variable was the total number of fractures per hen and phase. Age (continuous), hybrid (factor with 2 levels: LB, LSL) and their interaction were included as fixed effects, and hen nested in pen was used as a random effect. Model assumptions (normality of errors and homoscedasticity) were checked through graphical analysis of residuals. The final model was obtained by a stepwise backwards reduction using parametric bootstrap tests [package "pbkrtest" (17)] for model comparison and a $p > 0.05$ as the criterion for exclusion. The package "effects" (18) was used to calculate and display model estimates.

# RESULTS

## Radiographic Procedure

The mortality rate [percentage of dead hens/hens housed/4 weeks (19)] was 0.38% for focus birds ($n = 8$) and 0.87% for non-focus birds prior to the last radiographic timepoint at 61 weeks of age. No animals died during the procedure including: catching and transporting the birds from the housing system to the examination area, during suspension, or while returning them to the aviary system.

Following catching of the hens and placement in lairage crates, the procedure for each hen was approximately 60 s in duration beginning with removal from the crate, suspension, radiographic exposure and image collection, and finally returning the hen to the crate. During the first session, five out of 75 X-rays had to be repeated due to poor image quality (i.e., motion unsharpness). For the remaining sessions, approximately

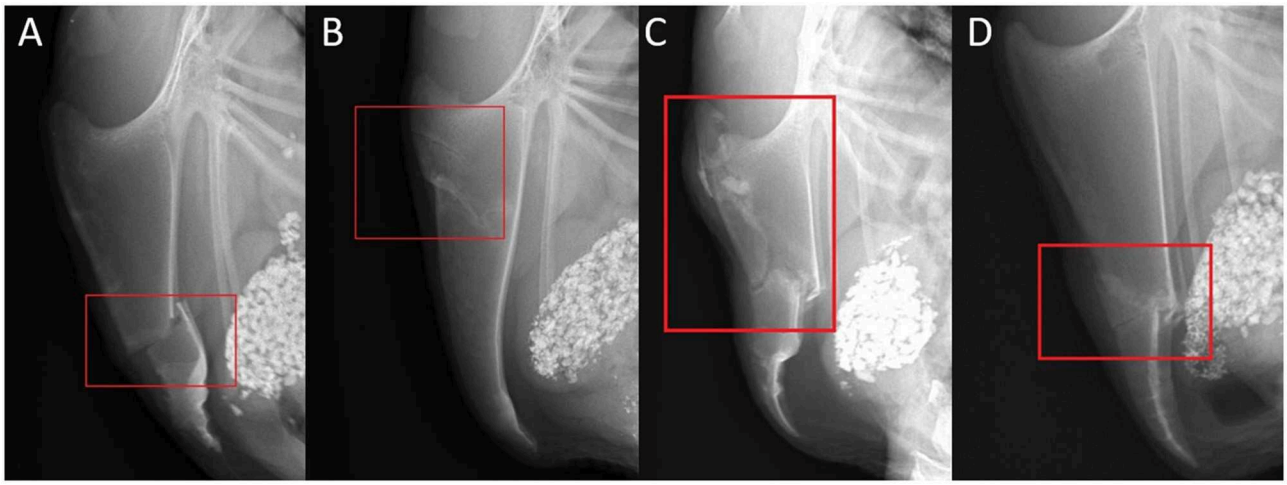


**FIGURE 3 |** Examples of fracture and lesion types and localizations. **(A)** Laterolateral view of a complete transverse keel bone fracture in the caudal third (localization C) with caudodorsal dislocation and angulation. **(B)** Laterolateral view of two incomplete oblique keel bone fractures in the cranial third (localization A) with a ventral superficial step formation and slight ventral angulation at the caudal fracture. **(C)** Laterolateral view of a comminuted keel bone fracture in the cranial and middle third (localization AB) with a ventrocranial dislocation and angulation of the caudal main fragment. **(D)** Laterolateral view of a butterfly keel bone fracture in the middle and the caudal third (localization BC) with ventral dislocation and angulation of the butterfly and caudal main fragment.

**TABLE 1 |** Mean, standard deviation (STD), minimum, and maximum value of total number of lesions per hybrid and time point.

| | LB | | | | LSL | | | |
|---|---|---|---|---|---|---|---|---|
| **Phase** | **Mean** | **STD** | **Min** | **Max** | **Mean** | **STD** | **Min** | **Max** |
| P1 | 0.1 | 0.3 | 0 | 2 | 0.1 | 0.3 | 0 | 1 |
| P2 | 0.3 | 0.5 | 0 | 2 | 0.4 | 0.7 | 0 | 2 |
| P3 | 0.5 | 0.8 | 0 | 3 | 0.9 | 0.9 | 0 | 3 |
| P4 | 1.1 | 1.1 | 0 | 5 | 1.4 | 1.1 | 0 | 4 |
| P5 | 1.8 | 1.3 | 0 | 5 | 2.0 | 1.2 | 0 | 4 |
| P6 | 2.4 | 1.6 | 0 | 7 | 2.3 | 1.2 | 0 | 5 |
| P7 | 2.8 | 1.8 | 0 | 9 | 2.5 | 1.2 | 0 | 6 |
| P8 | 3.3 | 2.1 | 0 | 11 | 2.7 | 1.2 | 1 | 6 |
| P9 | 3.6 | 2.2 | 0 | 13 | 2.9 | 1.2 | 1 | 6 |
| P10 | 3.8 | 2.2 | 0 | 13 | 3.1 | 1.2 | 1 | 6 |
| P11 | 4.0 | 2.4 | 0 | 15 | 3.2 | 1.2 | 1 | 6 |

**TABLE 2 |** Mean, standard deviation (STD), minimum, and maximum value of total number of fractures per hybrid and time point.

| | LB | | | | LSL | | | |
|---|---|---|---|---|---|---|---|---|
| **Phase** | **Mean** | **STD** | **Min** | **Max** | **Mean** | **STD** | **Min** | **Max** |
| P1 | 0.1 | 0.2 | 0 | 1 | 0.0 | 0.2 | 0 | 1 |
| P2 | 0.2 | 0.4 | 0 | 2 | 0.3 | 0.6 | 0 | 3 |
| P3 | 0.4 | 0.7 | 0 | 3 | 0.7 | 0.9 | 0 | 4 |
| P4 | 0.9 | 1.0 | 0 | 5 | 1.1 | 1.0 | 0 | 4 |
| P5 | 1.6 | 1.3 | 0 | 5 | 1.5 | 1.1 | 0 | 4 |
| P6 | 2.1 | 1.5 | 0 | 6 | 1.7 | 1.1 | 0 | 4 |
| P7 | 2.5 | 1.7 | 0 | 7 | 1.9 | 1.2 | 0 | 5 |
| P8 | 2.9 | 1.9 | 0 | 8 | 2.0 | 1.1 | 0 | 5 |
| P9 | 3.2 | 2.0 | 0 | 9 | 2.2 | 1.2 | 0 | 5 |
| P10 | 3.4 | 2.0 | 0 | 9 | 2.3 | 1.2 | 0 | 5 |
| P11 | 3.6 | 2.1 | 0 | 11 | 2.5 | 1.2 | 0 | 5 |

one radiograph per session had to be repeated due to poor image quality.

## Lesion Incidence

A total of 544 lesions including 422 fractures occurring on all 150 keel bones were identified; 99% of the hens had at least one keel bone lesion during the study period. Seventy-eight percent of the lesions were attributed to a fracture and 97% of the X-rayed animals had at least one KBF throughout the course of the study. Thirty percent of the hens had three KBF (mean ± SD for the whole database: 3.09 ± 1.80) at the end of the study with a maximum of 15 lesions (**Table 1**) and 11 fractures (**Table 2**) per keel bone observed. Keel bone lesions, which were not related to a fracture, hereafter referred to as non-fracture lesions, occurred in 22% of described lesions in this study. Of these, 39% were sclerotic areas, 39% angulations, 15% new bone formations, 6% indentations, and 1% other deformations. The number of new lesions per localization and hybrid is shown in **Table 3**.

## Fracture Types, Temporal Detection, and Development

The most frequent fracture types were transverse (45% of all fractures) and oblique (43% of all fractures). Comminuted fractures were present in 11% and butterfly fractures in 1% of all identified fractures. A considerable amount of fractures showed dislocation (34.8%), angulation (52.8%), or both (23.7%).

**TABLE 3 |** Absolute number of new lesions per localization for each phase and hybrid.

| | | | Number of new lesions per localization | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hybrid** | **Phase** | **# hens** | **A** | **A-B** | **A-B-C** | **A-D** | **B** | **B-C** | **C** | **C-E** | **D** | **E** | **Total** |
| LB | P1 | 75 | 2 | 1 | | 1 | | | 1 | | 1 | 1 | 7 |
| | P2 | 75 | 2 | | | | | | 8 | | 1 | | 11 |
| | P3 | 75 | 1 | | | | 2 | | 16 | | 1 | 1 | 21 |
| | P4 | 74 | 7 | | | | 3 | | 35 | | 1 | 6 | 52 |
| | P5 | 74 | 2 | 3 | 1 | | | | 32 | | 1 | 4 | 43 |
| | P6 | 74 | 1 | | | | | | 19 | | | 14 | 34 |
| | P7 | 74 | 4 | 2 | 1 | | 3 | | 23 | 1 | 1 | 9 | 44 |
| | P8 | 74 | 3 | 2 | | | 5 | | 14 | | 1 | 7 | 32 |
| | P9 | 73 | 2 | 2 | | | 1 | 1 | 11 | | 3 | 6 | 26 |
| | P10 | 72 | | 2 | | | 2 | | 8 | | 2 | 3 | 17 |
| | P11 | 72 | | | | 1 | 1 | | 10 | | 2 | 3 | 17 |
| LSL | P1 | 75 | | 3 | | | | | 4 | 1 | | 1 | 9 |
| | P2 | 75 | 1 | 1 | | | | | 16 | | | 3 | 21 |
| | P3 | 75 | 2 | 1 | | | | | 19 | 1 | 2 | 12 | 37 |
| | P4 | 75 | 3 | 2 | | | | | 25 | 1 | 1 | 18 | 50 |
| | P5 | 75 | 6 | 2 | | | | | 15 | 1 | | 5 | 29 |
| | P6 | 75 | 2 | 3 | | | 2 | 1 | 12 | 1 | | 4 | 25 |
| | P7 | 74 | 2 | 1 | | | 1 | 1 | 5 | | | 4 | 14 |
| | P8 | 74 | 5 | 3 | | | 1 | 1 | 5 | | | 3 | 18 |
| | P9 | 73 | 4 | 2 | | | 3 | | 4 | 1 | | 3 | 17 |
| | P10 | 72 | 2 | 1 | | | | | 4 | | 1 | 1 | 9 |
| | P11 | 70 | 7 | 2 | | | | | 1 | | | 1 | 11 |
| Total | | | 58 | 33 | 2 | 2 | 24 | 4 | 287 | 7 | 18 | 109 | 544 |

*Total number of hens and total number of new lesions are given for reference. One individual hen could be affected by multiple new lesions in the same or multiple localizations.*

Angulation occurred roughly equally in the dorsal (25%) and ventral (26%) directions. In about 4% of fractures, the direction of angulation changed between subsequent observation periods.

Most fractures occurred between 28 and 37 weeks of age (P3–P5; **Table 4**), with a peak during P4 when 45% of LB and 37% of LSL hens acquired a new fracture. During P4, as a percentage of all fractures within a genetic line, 17 and 20% were observed in LB and LSL, respectively (**Figure 4**). There was a peak in new transverse and oblique fractures as well as new comminuted and butterfly fractures between week 33 and 37 (P4, P5). Whereas, the occurrence of new transverse and oblique fractures seemed to decrease rather linearly after P4, the occurrence of butterfly and comminuted fractures was more variable (**Figure 5**, **Table 5**). The areas A, C, and E were affected by new lesions (fracture and non-fracture related lesions) mainly between week 31 and 33 (P4) (A: 17% of all lesions in A, C: 21% of all lesions in C, E: 22% of all lesions in E), whereas the occurrence of fractures in other localizations did not seem to be related to specific observation phases (**Figure 6**, **Table 4**).

Fifty-four percent of all lesions, which were initially not described as a fracture, fractured in the following observation period, leading to a total of 461 fractures. Forty-six percent of the initially described scleroses fractured in a later observation period.

In 84% of fractures, complete healing was diagnosed. Of these, 20% were already entirely healed at the time of detection and 43 and 22% healed within one or two observation periods, respectively. Less than 11% of the complete healed fractures required between 12 and 32 weeks until the fracture was healed as determined by radiography. In 16% of fractures, no healing could be detected.

In 76% of fractures, a callus was formed; in 50% a callus was already present at the time of fracture occurrence, whereas in 26% of new fractures the callus appeared at a later observation period (**Figure 7**). Of these, 26% (i.e., 7% of all fractures) showed a soft tissue swelling at the time of fracture detection. Of the 24% of the fractures without callus, the fracture gap completely disappeared over time and no step at the bone surface was visible in 13% of the cases, whereas the gap disappeared but a step at the bone surface was visible in a later observation time in 1% of all fractures. Nine percent manifested no fracture healing at all. The majority of the keel bones shortened during the observation period (65%). The length of the keel did not change in 9% and increased in 26%.

## Fracture Location

From all fractures, 62% were localized in section C, 15% in E, followed by sections A, AB, B, D, BC, and CE (**Figure 8**, **Table 4**). From all non-fracture lesions, 39% were localized in

**TABLE 4 |** Absolute number of new fractures per localization for each phase and hybrid.

| | | | Number of new fractures per localization | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Hybrid** | **Phase** | **# hens** | **A** | **A-B** | **A-B-C** | **A-D** | **B** | **B-C** | **C** | **C-E** | **D** | **E** | **Total** |
| LB | P1 | 75 | 1 | 1 | | | | | 1 | | 1 | | 4 |
| | P2 | 75 | 2 | | | | | | 6 | | 1 | | 9 |
| | P3 | 75 | 1 | | | | 1 | | 16 | | 1 | | 19 |
| | P4 | 74 | 4 | | | | 3 | | 31 | | 1 | 5 | 44 |
| | P5 | 74 | 2 | 3 | 1 | | | | 29 | | 1 | 3 | 39 |
| | P6 | 74 | 1 | | | | | | 18 | | | 10 | 29 |
| | P7 | 74 | 3 | 1 | 1 | | 1 | | 21 | 1 | 1 | 5 | 34 |
| | P8 | 74 | 2 | 1 | | | 2 | | 13 | | 1 | 7 | 26 |
| | P9 | 73 | 2 | 1 | | | 1 | 1 | 9 | | 1 | 3 | 18 |
| | P10 | 72 | | 1 | | | 2 | | 8 | | 1 | 2 | 14 |
| | P11 | 72 | | | | 1 | 1 | | 10 | | 2 | 3 | 17 |
| LSL | P1 | 75 | | | | | | | 2 | | | 1 | 3 |
| | P2 | 75 | | 1 | | | | | 16 | | | 2 | 19 |
| | P3 | 75 | 1 | 1 | | | | | 17 | 1 | | 5 | 25 |
| | P4 | 75 | 1 | | | | | | 22 | | 1 | 10 | 34 |
| | P5 | 75 | 3 | 2 | | | | | 14 | 1 | | 1 | 21 |
| | P6 | 75 | 1 | 2 | | | 2 | 1 | 11 | 1 | | | 18 |
| | P7 | 74 | 2 | | | | 1 | 1 | 4 | | | 3 | 11 |
| | P8 | 74 | 2 | 2 | | | 1 | | 4 | | | 1 | 10 |
| | P9 | 73 | 3 | 2 | | | 2 | | 4 | 1 | | 1 | 13 |
| | P10 | 72 | 1 | | | | | | 3 | | 1 | | 5 |
| | P11 | 70 | 7 | 2 | | | | | 1 | | | | 10 |
| Total | | | 39 | 20 | 2 | 1 | 17 | 3 | 260 | 5 | 13 | 62 | 422 |

*Total number of hens and total number of new fractures are given for reference. One individual hen could be affected by multiple new fractures in the same or multiple localizations.*

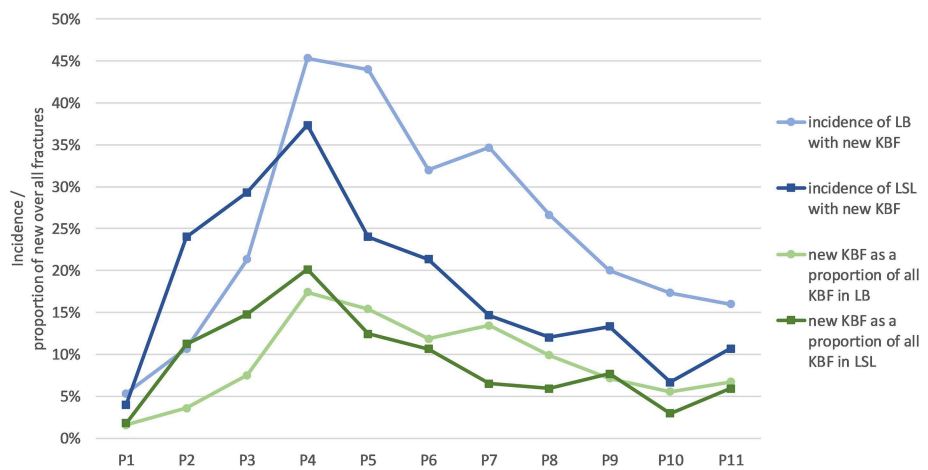


**FIGURE 4 |** Incidence of hens with new fractures per phase (blue lines) and new fractures in proportion to all fractures per phase (green lines) for LB (Lohmann Brown) and LSL (Lohmann Selected Leghorn) hens.

section E, 22% in C, 16% in A, 11% in AB, followed by B, D, CE, BC and AD (**Table 3**). The most common lesion types depending on location were: sclerosis in 38% of non-fracture lesions in section A, angulation in 92% of non-fracture lesions in section E, new bone formation in 56% of non-fracture lesions in section C, and indentations in 86% of non-fracture lesions in section AB.

Sixty-eight percent of KBF extended the entire height of the keel, running from the ventral to the dorsal or from the ventral to the cranial bone surface. Ninety-seven percent of all fractures

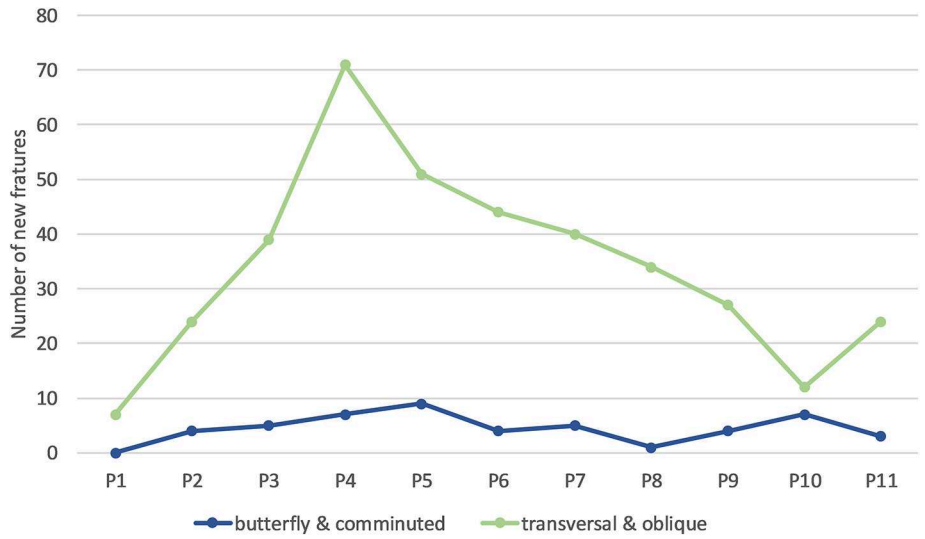


**FIGURE 5 |** Number of new keel bone fractures of different types occurring per phase across all hens. One individual hen could be affected by multiple new fractures of the same or different types.

in section A, 95% of all fractures in B, 71% of all fractures in AB, and 60% of all fractures in CE did not extend the entire height. All fractures in sections AD and ABC, 67% of all fractures in BC, 90% of all fractures in E, 79% of all fractures in C and 77% of all fractures in D did run the entire height.

Transverse and oblique fractures were mostly localized in section C (60%). Comminuted and butterfly fractures were predominantly localized in section C (73%), but also occurred in ABC (4%).

## Genetic Lines

In relation to genetic line, 95 and 99% of the LSL and LB animals, respectively, had at least one fracture. Sixty percent of all fractures were found in LB hens (and 40% in LSL). An average of 2.52 ± 1.20 (mean ± SD) and 3.63 ± 2.14 of fractures per keel bone were found for LSL and LB hens, respectively (**Table 2**). A maximum of 5 and 11 fractures per keel bone for LSL and LB hens, respectively, were visible. The increase in the total number of fractures per hen was steeper for LB hens than for LSL hens ($p_{hybrid*age} < 0.001$; **Figure 9**). Accordingly, the average number of KBF per hen was higher in LSL hens until 33 weeks of age (P4), whereas LB hens had more fractures than LSL hens in P5–P11 (**Table 2**).

## Soft Tissue Swelling

In 25% of the fractures, a soft tissue swelling was detected at the first time being observed. In 69% of comminuted fractures, a concurrent soft tissue swelling was present. Butterfly fractures showed soft tissue swelling in 25% and transverse and oblique fractures in 21% of the cases at the first time being observed.

# DISCUSSION

## Study Value

To the author's knowledge, the current manuscript is the first analysis of radiographed keel bone damage that includes detailed information regarding damage morphology and development along an entire production period of laying hens. The radiographic procedure was rapidly performed in the commercial environment and generated relatively high-quality images without accidental radiation exposure, thus we believe our protocol can effectively deliver accurate representations of the keel suitable for future research efforts. By combining objective assessment of individual facets of keel damage with the detail of repeated radiographic assessments over a 40 week period, our assessment protocol provided several findings that will improve understanding of KBF and its causes. We believe these benefits are afforded directly by the level of detail provided by radiography which would not be possible with other common methods of assessment, i.e., palpation or dissection.

## Fracture Incidence

For instance, a relatively surprising result that appears to conflict with earlier reports are the sheer number of hens that manifested fracture or damage of some type. In this study, 97.0% of the animals had at least one fracture and 99.3% had at least one lesion at the end of the study (61 weeks of age). Previous studies have suggested less frequent occurrence (5, 20–24) with typically 50–80% of surveyed hens manifesting keel fractures by the end of lay using palpation or dissection. As a potential explanation for the relatively higher frequency seen in the current study, our protocol allowed for recent, healed, and minor fractures to be diagnosed (7, 9) which could be missed with palpation and/or dissection. Alternatively, flock and facility differences could have played a role as our observations where conducted within a single barn, though previous efforts in the same barn with assessment by dissection also found a lower frequency of fracture (25). Nonetheless, our findings suggest the problem may be more severe than previously thought and highlight the need for reliable metrics when assessing KBF that can

**TABLE 5 |** Absolute number of new fractures per fracture type for each phase and hybrid.

| | | | Number of new fractures per fracture type | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Hybrid** | **Phase** | **# hens** | **Butterfly** | **Greenstick** | **Oblique** | **Comminuted** | **Transverse** | **Total** |
| LB | P1 | 75 | | | 2 | | 2 | 4 |
| | P2 | 75 | | | 1 | 3 | 5 | 9 |
| | P3 | 75 | | | 7 | 4 | 8 | 19 |
| | P4 | 74 | | 2 | 13 | 5 | 24 | 44 |
| | P5 | 74 | 2 | 1 | 7 | 4 | 25 | 39 |
| | P6 | 74 | | 3 | 14 | 2 | 10 | 29 |
| | P7 | 74 | | 2 | 15 | 3 | 14 | 34 |
| | P8 | 74 | | 2 | 12 | 1 | 11 | 26 |
| | P9 | 73 | | 2 | 8 | 2 | 6 | 18 |
| | P10 | 72 | 1 | | 8 | 4 | 1 | 14 |
| | P11 | 72 | | | 10 | 2 | 5 | 17 |
| LSL | P1 | 75 | | | | | 3 | 3 |
| | P2 | 75 | | | 9 | 1 | 9 | 19 |
| | P3 | 75 | | 1 | 9 | 1 | 14 | 25 |
| | P4 | 75 | | | 9 | 2 | 23 | 34 |
| | P5 | 75 | | | 9 | 3 | 9 | 21 |
| | P6 | 75 | | 1 | 7 | 2 | 8 | 18 |
| | P7 | 74 | 1 | 1 | 1 | 1 | 7 | 11 |
| | P8 | 74 | | | 7 | | 3 | 10 |
| | P9 | 73 | | 2 | 7 | 2 | 2 | 13 |
| | P10 | 72 | | | 1 | 2 | 2 | 5 |
| | P11 | 70 | | | 9 | 1 | | 10 |
| Total | | | 4 | 17 | 165 | 45 | 191 | 422 |

*Total number of hens and total number of new fractures are given for reference. One individual hen could be affected by multiple new fractures in the same or multiple localizations.*

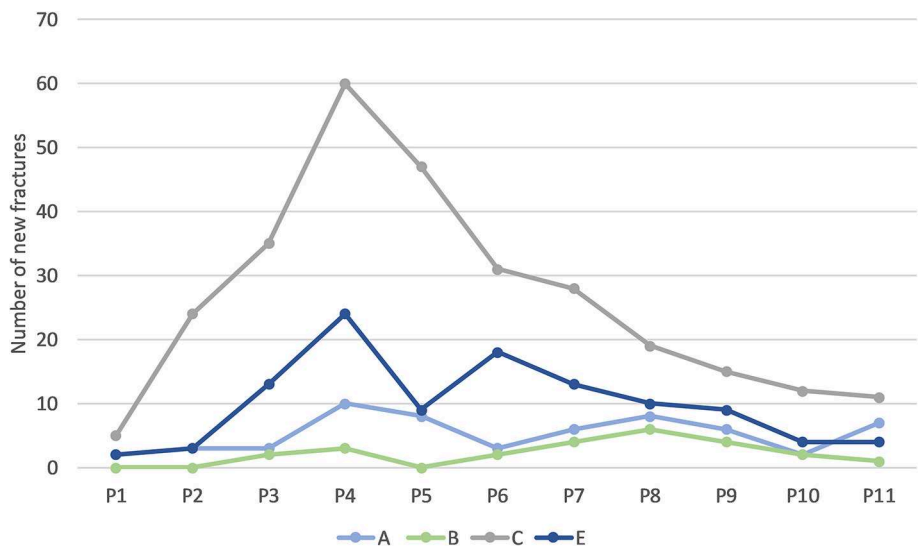


**FIGURE 6 |** Number of new lesions (including fractures and non-fracture lesions) in different bone areas occurring per phase across all hens. One individual hen could be affected by multiple new fractures in the same or in multiple areas.

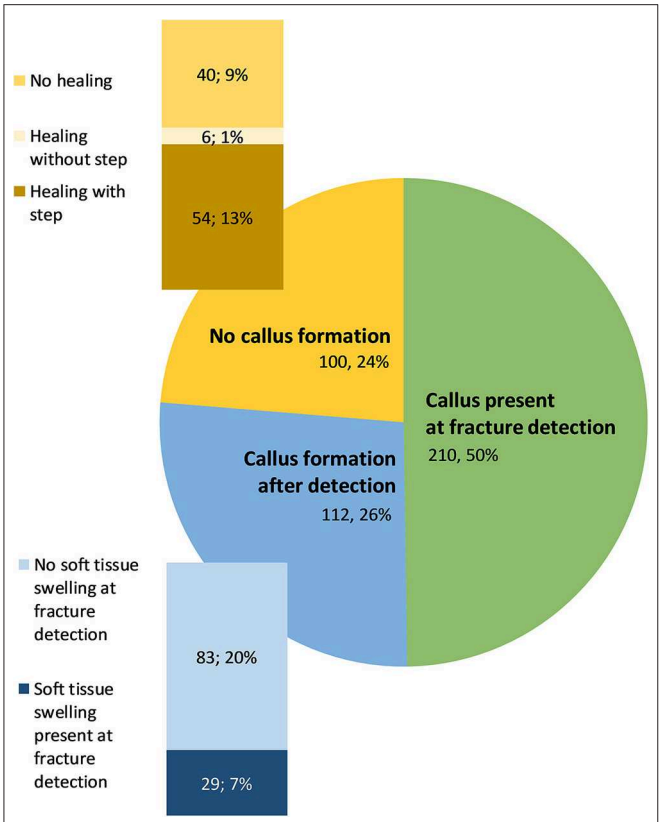


**FIGURE 7 |** Absolute numbers and proportion of fractures with callus present at fracture detection, after fracture detection, or forming no callus. Within the fractures without callus formation, the absolute number and of proportion of fractures with different healing processes is shown. Within the fractures with callus formation after detection, the absolute number and proportion of fractures with and without soft tissue swelling is given.

be compared across varying conditions and situations. Given that the radiographic procedure can be performed rapidly in both commercial and non-commercial environments to generate relatively high-quality images, we believe our protocol should be adopted for future efforts.

In our study, 76% of the fractures developed a callus which is one of the main features recognized during palpation. However, at the time of detection, only 49% of the fractures had callus formation and, in 24% of the fractures, no callus was visible of which 95% showed no soft tissue swelling. In the absence of palpable indicators of damage such as soft tissue swelling, crepitation, angulation/dislocation of fragments, and callus formation, diagnosing fractures by palpation is impossible, where especially acute fractures without soft tissue swelling and/or crepitation could be missed leading to a false negative result. Some of the non-fracture lesions were also associated with soft tissue swelling, which could be misinterpreted as a fracture by palpation, i.e., a false positive result. Clearly, radiography can be assumed to be far more sensitive to aspects of fracture than palpation providing substantial benefits to detection efforts. Radiography also proved to be effective in assessing damage that might not be possible by palpation because of location due to muscle mass especially in the cranial (A and B) and dorsal portions.

## Fracture Location and Type

In addition to specific features of KBF that our protocol could characterize, it also afforded the ability to distinguish the fracture types and location. Fractures on the keel bone were predominantly transverse and oblique and were localized in area C (caudal third), an area recognized as the most frequently affected (20, 23). Although this study was not intended to assess the causes of observed damage, characterizing KBF features in this manner could aid in this process and should be considered. For instance, a possible explanation could be that section C the keel bone has less stability due to the anatomically reduced diameter in contrast to areas A or B making the area more susceptible to fracture. However, this would not explain why the apices E and D were affected much less frequently than C. Another explanation to explain the differential frequencies of damage in these areas could be related to the muscling of the animals. On the keel bone, the flight muscles of the animals are attached laterally and large muscling in areas A and B might absorb external impact forces which occur in the case of a collision. As area C is less muscled (26), this section may lack the capacity to absorb external forces. Another explanation for the high rates of damage in section C may involve the presence of internal organs like the gizzard which lies directly dorsal to the keel and is relatively rigid with high resistance (27). As a force applied to the keel is absorbed by underlying compressive tissue such as air sacs, area C might be more susceptible to fracture during external impact due to the collision with the incompressible, stone-filled gizzard.

We are not able to provide a plausible explanation for the high prevalence of transverse and oblique fractures. Assuming that butterfly and comminuted fractures are mostly caused by an external impact, at least some of the transverse and oblique fractures might occur spontaneously, e.g., pathological fractures, a possibility raised previously (1). Pathological fractures might be expected with relatively strong muscles contracting against weak bone and are supported by the fact that these fractures frequently resembled fractures reported in mammals with primary or secondary hyperparathyroidism (28). It may be assumed that any bone damage is a combination of decreased bone strength and internally and externally applied forces, a possibility supported by observations of increased fracture susceptibility with decreased keel bone mineral density (29).

## Fracture Development

Most keel bone fractures in this study occurred when hens were 31–33 weeks of age, a period just after peak of lay where hens have been laying eggs regularly for approximately 10 weeks. When reaching sexual maturity at 16–18 weeks of lay, a hen's bone metabolism undergoes dramatic changes: production of structural bone ceases (30), the elasticity of the bones decreases (31), and calcium retention efficiency increases (32). The loss of bone elasticity might be insufficiently compensated by the increase in calcium retention around the age of 31–33 weeks, leading to an increase in the incidence of fractures. The

subsequent decrease in new fractures during the following weeks could partly be explained by the increase of calcium retention, which would solidify the bone. Alternatively, the high incidence of new fractures at 31–33 weeks of age results in callus formation, which deforms the bone and makes it thicker. This would not only explain the lower incidence of KBF for hens older than 33 weeks but would also explain the higher incidence of butterfly and comminuted fractures, as more severe trauma would be needed to cause these types of fracture.

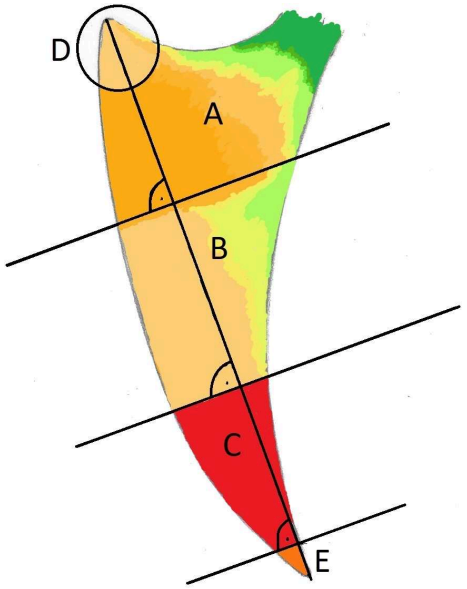


**FIGURE 8 |** Subjective schematic visualization of the spatial frequency of new keel bone fractures: Low (green), moderate (yellow) and high (red) frequency. **(A)** Cranial third, **(B)** middle and **(C)** caudal third. The apices are labeled **D** (cranial; $r = 15$ mm) and E (caudal 1/5 of **C**).

Another hypothesis to explain the higher incidence of KBF at the age of 31–33 weeks would be that, after moving the hens from the rearing to the laying barn, hens need to adapt to their new environment and might use the lower perches of the aviary less than the upper perches. Indeed, use of the upper perches increases with increasing age (13) and was found to be a risk factor for KBF (20). After a linear increase in KBF incidence leading to a peak at P4, this will reduce again due to a lower use of the perches. Indeed, the prevalence of KBF was then found to be high, and hens with keel bone lesions showed reduced mobility in moving between tiers and associated perches (13). Further research is needed to confirm these hypotheses, though we believe our methodology contributes to this effort.

Fractures located in the region of the breast muscles (A and B, used during flight) as well as fractures in the region of the keel bone exposed while approaching the perch (C and E) would be expected to cause more pain than fractures in region D, as more forces are applied. Comminuted fractures and fractures with dislocated fragments leading to a long healing period were also suspected to be of greater importance regarding the welfare of the hen when compared to smaller and non-dislocated fractures at the ventral aspect of the keel bone. These expectations were not evaluated in our study, though we believe the development of the described methodology is a critical first step toward those

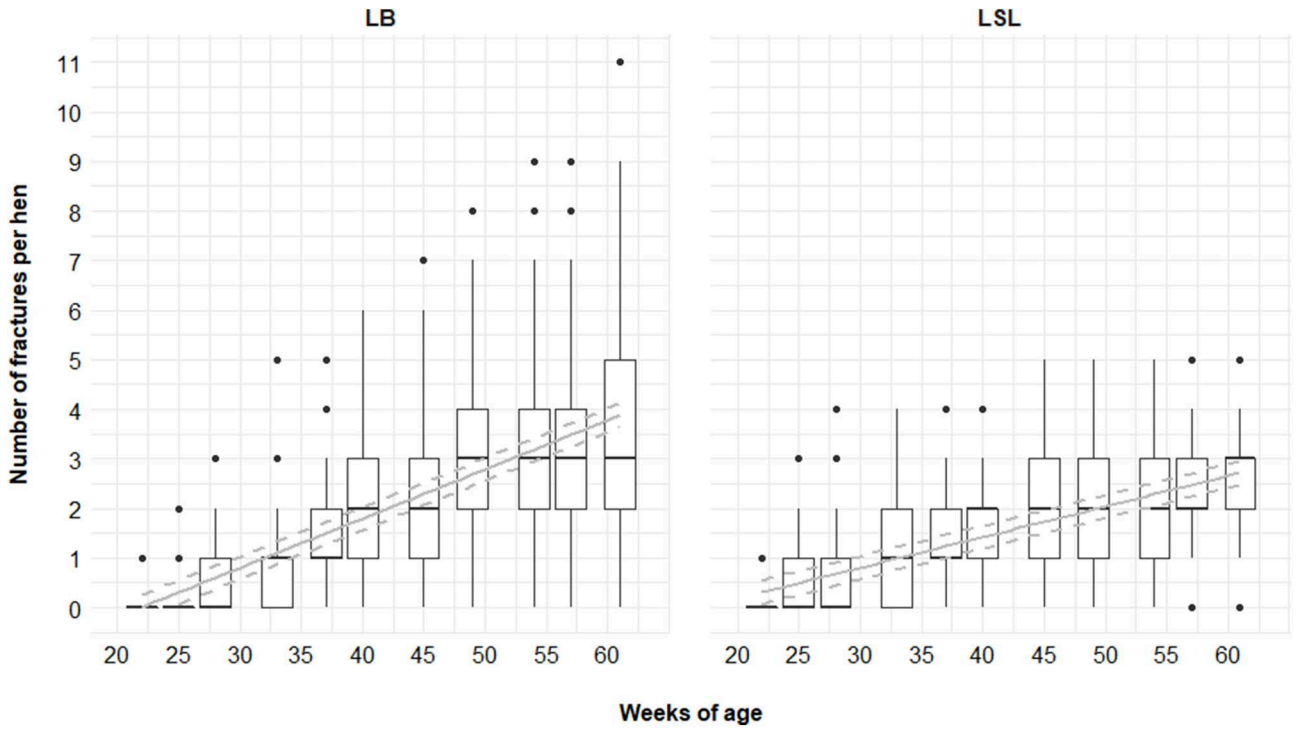


**FIGURE 9 |** Total number of fractures per hen and phase for LB (Lohmann Brown) and LSL (Lohmann Selected Leghorn) hens. Boxplots show medians, interquartile and absolute ranges of raw data plus outliers. Solid lines represent estimated means, dashed lines indicate the 95% confidence interval.

goals. Our effort was also unique in allowing for longitudinal observations of damage in a comparable manner. Radiography allowed multiple age- and hen-specific images to be overlaid and features of interest to be compared. By making these comparisons within hens over time, our efforts found the duration of healing ranged from 0 (radiographically already healed at detection) to 36 weeks with 85% of fractures healing within 7 weeks, results that are in accordance with Richards et al. (9). Fractures that did not heal or required extended time to heal, often lasting several months without evidence of healing, are also a known concern in mammals. Explanations for delayed or absent fracture healing are missing healing stimuli either due to a lack or cloying (micro-) motion at the fracture ends or a too large fracture gap due to extensive fragment dislocation (33). Additionally, decreased primary osteoclastic and osteoblastic activity might influence fracture healing. All these conditions can lead to atrophic or hypertrophic non-union of fragments. The concept of fracture treatment involves fragment repositioning, stabilization/fixation, and restriction of motion, measures which have not been an option in our quasi-commercial study setting or that of a standard production environment. Spontaneous fracture healing in wild animals is limited by persisting motion and limping. In analogy to fracture healing in wild animals, spontaneous fracture healing in our focus animals mostly lead to mal-union and shortening of the keel bone (34).

### Non-fracture Lesions

Fifty-five percent of the non-fracture lesions and 45% of the sclerosis developed in a later observation period to a fracture. Other non-fracture lesions were angulations, predominantly in section E, and indentations, predominantly in section AB. The occurrence and development of such lesions might support the hypothesis of the presence of decreased bone strength and therefore high susceptibility to any kind of damage. In this context, indentations might be the result of chronic external pressure on the keel applied when the hens are sitting on the perch (2).

### Study Limitation

In the current study, only a laterolateral radiograph of the keel bone was performed, which is a major shortcoming as an accurate image interpretation should involve at least two projections at 90° to each other. The authors are aware that a single projection would lead to some lesions (e.g., deviations) being missed, underestimated (direction and amount of dislocation and angulation) or misinterpreted [fracture gap, callus formation, (35)]. Even though two projections at an angle of 90° to each other is necessary, preliminary efforts in a pilot study demonstrated that a craniocaudal tangential or ventrodorsal projection proved ineffective due to superimposition.

## CONCLUSIONS

The described radiographic protocol for keel bone lesions is suitable for longitudinal on-site examinations. Keel bone fractures appear more frequent than reported in earlier studies which we believe relates to our protocol's superior ability to assess damage. Further investigations should be conducted to understand the clinical significance (e.g., activity, productivity, pain) as well as the cause for damage using the described technique generating detailed representations of keel damage.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

The use of radiography for animal experimentation was reviewed and approved by the Swiss radiation authority (Approval number BE-03222.41.013). Approval for use of experimental animals was obtained from the Veterinary Office of the Canton of Bern in Switzerland (approval number BE31/15). The experiment complied with Swiss regulations regarding the treatment of experimental animals.

## AUTHOR CONTRIBUTIONS

SB produced the radiographs, analyzed data, was the principal developer of the scoring-system, and was the principal author of the manuscript. CR was the principal organizer of the daily operations for the parallel study as part of a doctoral research program, provided important information on study design, and performed the statistics used for the tables and **Figure 9**. MT was principal supervisor of the parallel project and was also the recipient for the associated funding. UG supervised this project, gave major inputs regarding radiographic evaluation and development of the scoring system, and was responsible for radiographic quality and radiation protection. All authors reviewed the manuscript and approved the submitted version.

## FUNDING

This project was funded by the Federal Food Safety and Veterinary Office FSVO (project number 2.15.05) with additional support for radiographic equipment by the Eva Husi and Haldimann Foundations. The funding partners had no possibility to influence and contribute to the data collection, analysis and interpretation and to manuscript writing.

## ACKNOWLEDGMENTS

We thank the tireless helpers Edi Burkhard, Selina Mühlemann and Suzanne Petit in catching the birds and taking the radiographs and Claudine Petrig for providing Esmeralda for **Figure 1**.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00129/full#supplementary-material

**Data Sheet S1 |** Raw data used for the study.

## REFERENCES


1. Harlander-Matauschek A, Rodenburg TB, Sandilands V, Tobalske BW, Toscano MJ. Causes of keel bone damage and their solutions in laying hens. *Worlds Poult Sci J.* (2015) 71:461–72. doi: 10.1017/S0043933915002135
2. Pickel T, Schrader L, Scholz B. Pressure load on keel bone and foot pads in perching laying hens in relation to perch design. *Poult Sci.* (2011) 90:715–24. doi: 10.3382/ps.2010-01025
3. Fleming RH, McCormack HA, McTeir L, Whitehead CC. Incidence, pathology and prevention of keel bone deformities in the laying hen. *Br Poult Sci.* (2004) 45:320–30. doi: 10.1080/00071660410001730815
4. Petrik MT, Guerin MT, Widowski TM. On-farm comparison of keel fracture prevalence and other welfare indicators in conventional cage and floor-housed laying hens in Ontario, Canada. *Poult Sci.* (2015) 94:579–85. doi: 10.3382/ps/pev039
5. Wilkins LJ, McKinstry JL, Avery NC, Knowles TG, Brown SN, Tarlton J, et al. Influence of housing system and design on bone strength and keel bone fractures in laying hens. *Vet Rec.* (2011) 169:414. doi: 10.1136/vr.d4831
6. Regmi P, Deland TS, Steibel JP, Robison CI, Haut RC, Orth MW, et al. Effect of rearing environment on bone growth of pullets. *Poult Sci.* (2015) 94:502–11. doi: 10.3382/ps/peu041
7. Casey-Trott T, Heerkens JLT, Petrik M, Regmi P, Schrader L, Toscano MJ, et al. Methods for assessment of keel bone damage in poultry. *Poult Sci.* (2015) 94:2339–350. doi: 10.3382/ps/pev223
8. Hecht S. *Röntgendiagnostik in der Kleintierpraxis*. 2nd ed. Stuttgart: Schattauer GmbH (2012). doi: 10.1055/b-005-148989
9. Richards GJ, Nasr MA, Brown SN, Szamocki EMG, Murrell J, Barr F, et al. Use of radiography to identify keel bone fractures in laying hens and assess healing in live birds. *Vet Rec.* (2011) 169:279. doi: 10.1136/vr.d4404
10. Širovnik J, Toscano MJ. Restraining laying hens for radiographic diagnostics of keel bones. In: *Proceedings of the 10th European Symposium on Poultry Welfare*. Ploufragan (2017). p. 162. Available online at: http://www.wpsa.com/index.php/publications/wpsa-proceedings/2017/x-espw
11. Clark WD, Cox WR, Silversides FG. Bone fracture incidence in end-of-lay high-producing, noncommercial laying hens identified using radiographs. *Poult Sci.* (2008) 87:1964–970. doi: 10.3382/ps.2008-00115
12. Rufener C, Baur S, Stratmann A, Toscano MJMJ. A reliable method to assess keel bone fractures in laying hens from radiographs using a tagged visual analogue scale. *Front Vet Sci.* (2018) 5:124. doi: 10.3389/fvets.2018.00124
13. Rufener C, Abreu Y, Asher L, Berezowski JA, Maximiano F, Stratmann A, et al. Keel bone fractures are associated with individual mobility of laying hens in an aviary system. *Appl Anim Behav Sci.* (2019) 217:48–56. doi: 10.1016/j.applanim.2019.05.007
14. Rufener C, Baur S, Stratmann A, Toscano MJ. Keel bone fractures affect egg laying performance but not egg quality in laying hens housed in a commercial aviary system. *Poult Sci.* (2018) 98:1589–600. doi: 10.3382/ps/pey544
15. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *J Stat Softw.* (2015) 67:1–48. doi: 10.18637/jss.v067.i01
16. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing (2017). Available online at: http://www.r-project.org/
17. Halekoh U, Højsgaard S. A kenward-roger approximation and parametric bootstrap methods for tests in linear mixed models - the R package pbkrtest. *J Stat Softw.* (2014) 59:1–30. doi: 10.18637/jss.v059.i09
18. Fox J. Effect displays in R for generalised linear models. *J Stat Softw.* (2003) 8:1–27. doi: 10.18637/jss.v008.i15
19. Aerni V, Brinkhof MWG, Wechsler B, Oester H, Fröhlich E. Productivity and mortality of laying hens in aviaries: a systematic review. *Worlds Poult Sci J.* (2005) 61:130–42. doi: 10.1079/WPS200450
20. Heerkens JLT, Delezie E, Rodenburg TB, Kempen I, Zoons J, Ampe B, et al. Risk factors associated with keel bone and foot pad disorders in laying hens housed in aviary systems: table 1. *Poult Sci.* (2016) 95:482–8. doi: 10.3382/ps/pev339
21. Käppeli S, Gebhardt-Henrich S, Fröhlich E, Pfulg A, Stoffel MH. Prevalence of keel bone deformities in swiss laying hens. *Br Poult Sci.* (2011) 52:531–6. doi: 10.1080/00071668.2011.615059
22. Riber AB, Hinrichsen LK. Keel-bone damage and foot injuries in commercial laying hens in Denmark. *Anim Welf.* (2016) 25:179–84. doi: 10.7120/09627286.25.2.179
23. Wilkins LJ, Brown SN, Zimmerman PH, Leeb C, Nicol CJ. Investigation of palpation as a method for determining the prevalence of keel and furculum damage in laying hens. *Vet Rec.* (2004) 155:547–9. doi: 10.1136/vr.155.18.547
24. Toscano MJ, Booth F, Wilkins LJ, Avery NC, Brown SB, Richards G, et al. The effects of long (C20/22) and short (C18) chain omega-3 fatty acids on keel bone fractures, bone biomechanics, behavior, and egg production in free-range laying hens. *Poult Sci.* (2015) 94:823–35. doi: 10.3382/ps/pev048
25. Stratmann A, Fröhlich EKF, Gebhardt-Henrich SG, Harlander-Matauschek A, Würbel H, Toscano MJ. Modification of aviary design reduces incidence of falls, collisions and keel bone damage in laying hens. *Appl Anim Behav Sci.* (2015) 165:112–23. doi: 10.1016/j.applanim.2015.01.012
26. Sy M. Funktionell-anatomische untersuchungen am vogelflügel. *J für Ornithol.* (1936) 84:199–296. doi: 10.1007/BF01906709
27. Svihus B. The gizzard: function, influence of diet structure and effects on nutrient availability. *Worlds Poult Sci J.* (2011) 67:207–23. doi: 10.1017/S0043933911000249
28. Tomsa K, Glaus T, Hauser B, Flückiger M, Arnold P, Wess G, et al. Nutritional secondary hyperparathyroidism in six cats. *J Small Anim Pract.* (1999) 40:533–9. doi: 10.1111/j.1748-5827.1999.tb03015.x
29. Candelotto L, Stratmann A, Gebhardt-Henrich SG, Rufener C, van de Braak T, Toscano MJ. Susceptibility to keel bone fractures in laying hens and the role of genetic variation. *Poult Sci.* (2017) 96:3517–528. doi: 10.3382/ps/pex146
30. Hudson HA, Britton WM, Rowland GN, Buhr RJ. Histoomorphometric bone properties of sexually immature and mature white leghorn hens with evaluation of fluorochrome injection on egg production traits. *Poult Sci.* (1993) 72:1537–547. doi: 10.3382/ps.0721537
31. Schmid BA. Bestimmung des Verknöcherungsverlaufs des Brustbeins von schnell und langsam wachsenden Masthühnern [Dissertation]. (2008) Available online at: http://opus.uni-hohenheim.de/volltexte/2008/298/
32. Scott TA, Balnave D. Influence of temperature, dietary energy, nutrient concentration and self-selection feeding on the retention of dietary energy, protein and calcium by sexually-maturing egg-laying pullets. *Br Poult Sci.* (1991) 32:1005–016. doi: 10.1080/00071669108417425
33. Goodship AE, Kenwright J. The influence of induced micromovement upon the healing of experimental tibial fractures. *J Bone Joint Surg Br.* (1985) 67:650–5. doi: 10.1302/0301-620X.67B4.4030869
34. Kirberger RM, Keet DF, Wagner WM. Radiologic abnormalities of the appendicular skeleton of the lion (*Panthera leo*): incidental findings and *Mycobacterium bovis*-induced changes. *Vet Radiol Ultrasound.* (2006) 47:145–52. doi: 10.1111/j.1740-8261.2006.00121.x
35. Vogl TJ, Reith W, Rummeny EJ. *Diagnostische und Interventionelle Radiologie*. Berlin-Heidelberg: Springer-V. (2011). doi: 10.1007/978-3-540-87668-7


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 27 March 2020
doi: 10.3389/fvets.2020.00128

# Effects of Maternal Stress on Measures of Anxiety and Fearfulness in Different Strains of Laying Hens

*Mariana R. L. V. Peixoto*[1], *Niel A. Karrow*[1], *Amy Newman*[2] *and Tina M. Widowski*[1]*

[1] *Department of Animal Biosciences, University of Guelph, Guelph, ON, Canada,* [2] *Department of Integrative Biology, University of Guelph, Guelph, ON, Canada*

Maternal stress can affect the offspring of birds, possibly due to hormone deposition in the egg. Additionally, phenotypic diversity resulting from domestication and selection for productivity has created a variety of poultry lines that may cope with stress differently. In this study, we investigated the effects of maternal stress on the behavior of different strains of laying hens and the role of corticosterone as its mediator. For this, fertilized eggs of five genetic lines—two brown (Brown 1 and 2), two white (White 1 and 2), and one pure line White Leghorn—were reared identically as four flocks of 27 birds (24F: 3M) per strain. Each strain was equally separated into two groups: Maternal Stress ("MS"), where hens were subjected to a series of daily acute psychological stressors for 8 days before egg collection, and "Control," which received routine husbandry. Fertile eggs from both treatments were collected at three different ages forming different offspring groups that were treated as replicates; additional eggs from Control were injected either with corticosterone diluted in a vehicle solution ("CORT") or just "Vehicle." Eggs from each replicate were incubated and hatched, and offspring ($N$ = 1,919) were brooded under identical conditions. To measure the effects of maternal stress on anxiety and fear-like behavior, offspring were subjected to a social isolation test (SI) between 5 and 10 days of age and a tonic immobility test (TI) at 9 weeks of age. Compared to Control, MS decreased the number of distress vocalizations emitted by White 2 in SI. No effects of MS were observed in TI, and no effects of CORT were observed in any tests. Overall, brown lines vocalized more in SI and remained in TI for a longer duration than white strains, suggesting genetic differences in fear behavior. Females vocalized more than males in TI and showed a trend toward significance for the same trait in SI. Overall, results suggest that the effects of maternal stress on fearfulness are not directly mediated by corticosterone. Moreover, it highlights behavioral differences across various strains of laying hens, suggesting that fear responses are highly dependent on genotype.

**Keywords: corticosterone, layer breeder, fear, anxiety, genetics, chicken**

OPEN ACCESS

***Edited by:***
*Dana L. M. Campbell, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia*

***Reviewed by:***
*Andrew M. Janczak, Norwegian University of Life Sciences, Norway*
*Elske N. De Haas, Utrecht University, Netherlands*

***Correspondence:**
*Tina M. Widowski*
*twidowsk@uoguelph.ca*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *10 December 2019*
***Accepted:*** *20 February 2020*
***Published:*** *27 March 2020*

***Citation:***
*Peixoto MRLV, Karrow NA, Newman A and Widowski TM (2020) Effects of Maternal Stress on Measures of Anxiety and Fearfulness in Different Strains of Laying Hens. Front. Vet. Sci. 7:128. doi: 10.3389/fvets.2020.00128*

## INTRODUCTION

Maternal stress can impact offspring physiology, behavior, and cognition (1–3). Its effects are highly dependent on the intensity, timing of exposure, and type of stressor experienced by the mother (4–6). More specifically, impacts on offspring behavior are evident across taxa [avian (7); mammals (1, 8); reptiles (9)], and at the neurological scale, maternal stress has been linked to structural

and functional changes in the limbic system and prefrontal cortex of rats (1), and to changes in gene expression in the hypothalamus of chickens (10). These brain areas are involved in the mediation of fear and anxiety, social and cognitive processes, and working memory of mammals and birds (11, 12). Maternal stress may have long-term impacts on how an animal responds to its environment. For example, in laying hens, females subjected to an unpredictable food restriction schedule had chicks that stayed longer in tonic immobility (TI), a measure of fearfulness, and were less competitive for access to food in a novel environment than the offspring of control birds (13). Similarly, the offspring of female quails stressed during egg production displayed more anxiety-like behaviors, such as an increased occurrence of distress calls during emergence and open field tests, and when isolated from conspecifics (14).

Cottrell and Seckl (15) proposed two major hypotheses to explain the association between maternal stress and postnatal effects on offspring: fetal malnutrition and overexposure to glucocorticoid hormones. More recently, studies in avian species have shown that maternal stress can also be linked to the increase in other biological components in the egg, such as androgens (16), thyroid hormones (17), antioxidants (18), and immunoglobulins (19). Nevertheless, although glucocorticoid hormones are not a synonym for "stress" (20), corticosterone remains as one of the most analyzed mediators of maternal stress in the literature due to their pleiotropic role in regulating physiological responses to the environment and in the development and maturation of vital organs [reviewed in (6, 16, 21)]. Moreover, the hypothalamus–pituitary–adrenal (HPA) axis of chickens, responsible for corticosterone production, becomes functional between the 14 and 16th day of incubation and might also be affected by maternal hormone deposition (22). The effects of corticosterone on the behavior of the offspring are, however, inconsistent and appear to depend on delivery method and species, possibly being related only to metabolic and developmental processes (23). For example, although corticosterone injections into fertile eggs and implants to female chickens were linked to an increase in the duration of TI in the offspring (24, 25), injections in yellow-legged gulls had no effects in the same test (26). Moreover, corticosterone injections decreased the offspring's ability to learn (27), compete for a wormlike object (24), and increased aggressiveness (28) in layer chickens.

As evidenced above, two experimental models are commonly used to increase corticosterone levels in the egg: a maternal model in which the adult female is exposed to stressors (either directly or through corticosterone injections or implants) and a pharmacological model that manipulates the egg. The maternal model might be considered more holistic as hormone or stress treatments integrate with other maternal elements that might also affect embryonic development (29). However, it precludes a specific control of the quantity of hormone reaching the embryo (30). Conversely, egg manipulation allows the study of exposure to an exact dose of specific hormone but relies on the use of an invasive injection procedure that can be harmful to the embryo (31, 32). Furthermore, hormonal responses are generally dose-dependent, and the actual concentrations deposited by the mother into the egg during development remain unknown (16, 33).

Similarly unknown is the relationship between maternal stress and genetics. Although no previous studies have tested multiple strains of commercial layers simultaneously, the levels of susceptibility to maternal stress may vary across different genotypes. A positive correlation between the concentration of corticosterone in layer breeders and the occurrence of an anxiety-like behavior in the offspring was observed in a white genetic hybrid but not in a brown hybrid (34). Furthermore, it has been found that adult brown and white strains of laying hens have distinct behavioral and physiological responses to stress (34–36); and comparisons between offspring of White Leghorns and their ancestor, the red jungle fowl, revealed that in response to maternal stress, only the White Leghorn chickens displayed decreased learning abilities and differences in gene expression in the hypothalamus and pituitary, suggesting that genetic selection may have increased maternal stress susceptibility (37).

The main goal of this study was to investigate whether the effects of maternal stress on offspring fear- and anxiety-like behavior differ across genetic lines of laying hens. For this, five strains of breeder hens were subjected to two stress models: one that involved subjecting the breeders to acute psychological stressors and another that involved egg injections of corticosterone. Using these treatments, we sought to decouple the role of corticosterone from the broader maternal milieu during maternal stress. We predicted that injections would affect all strains, acting as a positive control treatment regardless of genetics, and that the effects of Maternal Stress would vary according to the natural stress susceptibility of each strain.

## MATERIALS AND METHODS

The birds used in this study were treated in accordance with the Canadian Council on Animal Care, and all procedures were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #1946). All the strains presented herein were anonymized as required by the genetics companies that donated the parent stock.

### Parent Stock: Management

A total of 2,600 fertilized eggs of five strains of parent stock were provided by two commercial genetics companies (Brown 1 and White 1 from company 1; Brown 2 and White 2 from company 2; each company donated 360 female line eggs and 64 male eggs per strain) and the University of Guelph's Arkell Poultry Research Station (pure line White Leghorn). To guarantee similar experiences, eggs from all strains were collected from grandparent hens that were between 40 and 50 weeks of age. Eggs and chicks were subjected to identical incubation and husbandry conditions, as previously described (38). Chicks were wing banded at hatch, and each strain was equally distributed into 4 parent flocks that were placed in 2 rooms containing 10 pens of 27 birds (24 females and 3 males) each (see **Supplementary Figure 1**). Pens (3.7 $m^2$) were enriched with pine shavings, one elevated perch and one lower perch, totaling a perch space of 12.8 cm/bird/pen. At 18 weeks, five

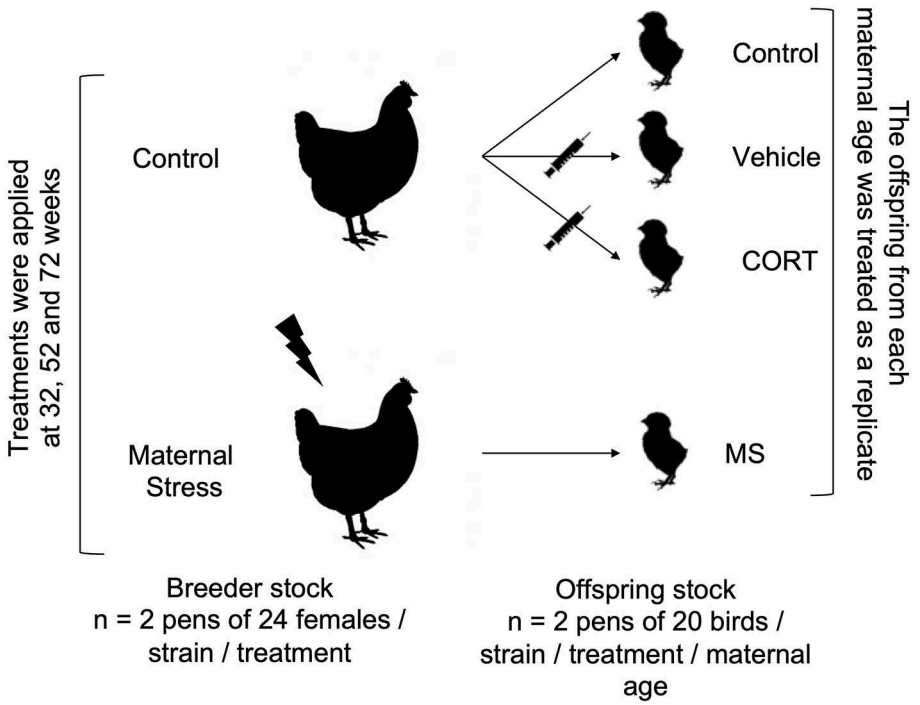


**FIGURE 1 |** Experimental design. Treatments (Control and Maternal Stress) were applied to each strain (Commercial Brown 1 and 2, Commercial White 1 and 2, and Pure Line White Leghorn) of the breeder flocks at three different ages (32, 52, and 72 weeks). The offspring of each maternal age were statistically treated replicates.

nest boxes were added to each one of the pens. Chickens from different pens were visually separated from each other and did not interact at any moment. Apart from routine husbandry, all human interaction was avoided to prevent possible habituation.

## Parent Stock: Experimental Design

Treatments and egg collection were performed at 32, 52, and 72 weeks of age. To form the offspring groups, equal numbers of fertile eggs (sampled over time, preference given to recent over old) from all parent flocks were incubated 1 day after the end of stressors, and the offspring flocks from each maternal age were treated as replicates (**Figure 1**). This experimental design allowed us to work with a larger sample size, but it also resulted in replicates confounded with incubatory settings, chick transfer and placement from the incubator to pens, and egg composition, since the nutritional value of the egg changes as a hen ages (39).

## Parent Stock: Control and Maternal Stress Treatments

Each flock of breeders was randomly assigned to either Control or Maternal Stress ("MS") treatments with two replicate flocks per strain and treatment. Regular husbandry was strictly adhered to for the Control groups, while the females of the MS flocks were subjected to daily sessions of acute psychological stress procedures that were selected based on their ability to increase plasma corticosterone concentration in avian species (see references for each test and species below). Since the average time window for egg production from the beginning of vitellogenesis until laying is 8 days, each MS flock received a minimum of 8 consecutive days of stressors before the beginning of egg collection.

Hens from the MS flocks were subjected to each of the following procedures: (1) Hens were equally distributed into two plastic crates (89 cm long × 60 cm wide × 26 cm high; 12 hens/crate), followed by 15 min of transportation [**Figure 2A**, laying hen: (40)]. (2) Hens were individually removed from their home pens and placed inside a cloth bag located in a nearby room for 10 min of physical restraint [**Figure 2B**, laying hen: (41)]. (3) Hens were crated into two groups of 12 birds, transported to an empty room 400 m away from their home pen and transferred to a test arena (100 cm long × 100 cm wide × 200 cm high) constructed of solid white panels with two doors located on opposite walls and two LED lights on the ceiling for 30 min. In the arena, hens were exposed to three simulations of a predator attack (30 s/each) using the silhouette of a sparrow-hawk made of black cardboard (35 cm long × 50 cm wide) [**Figures 2C,D**, great tit: (42)]. (4) Hens were crated and transported to the test arena for 15 min. An air horn was blown for 3 s at 5-min intervals [Japanese quail: 14; European starling: (43)]. (5) Hens were crated and transported to the test arena for 30 min with a different strain [laying hen: (44)]. All birds were immediately returned to their home pens after each stress session. Overall, sessions respected the following criteria: (1) Flocks were subjected to one stressor a day. (2) Stressors and egg collection were performed until the total number of eggs necessary for incubation had been collected. (3) To avoid a decrease in the physiological response to stressors due to repeated exposure, the minimum interval between the

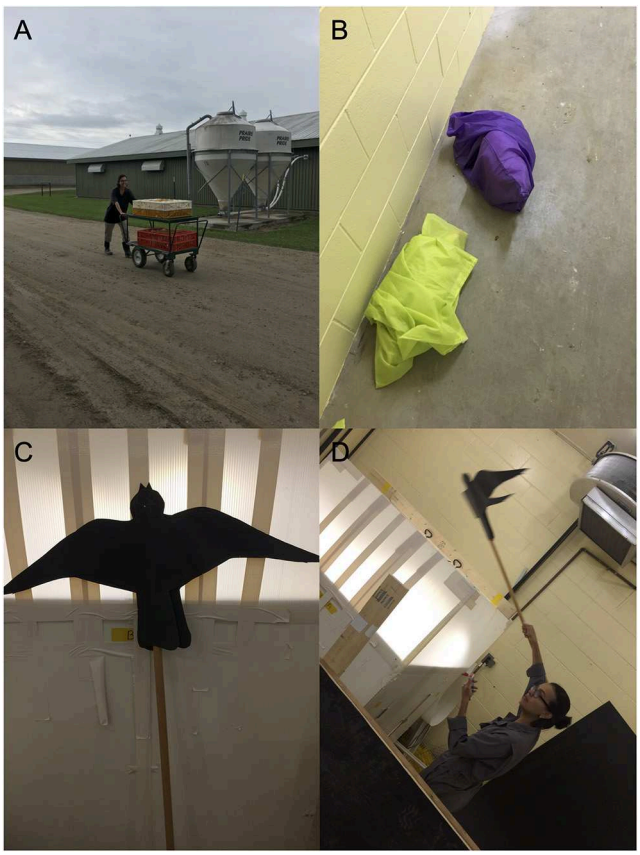


**FIGURE 2 |** Stressors used in the MS treatment. **(A)** Breeder hens were crated and transported around the research facility for 15 min. **(B)** Physical restraint in a cloth bag. **(C)** The silhouette of a sparrow-hawk made of black cardboard (35 cm long × 50 cm wide). **(D)** Layer breeders were transferred to a test arena (100 cm long × 100 cm wide × 200 cm high); three simulations of a predator attack were performed (All photos used with permission of subjects).

application of the same stressor was 4 days. (4) Sessions ran randomly from 9:00 to 16:00 h.

## Parent Stock: Vehicle and CORT Treatments

The CORT treatment aimed to increase the concentration of corticosterone in fertilized eggs from breeder hens. According to previous studies, the basal level of corticosterone in laying hens ranges from 0.3 to 5 ng/ml (45), reaching 30 ng/ml in response to stress (46). The concentration of corticosterone in egg yolks has been previously reported to range from 0.77 to 2.8 ng/g in Hy-Line Brown (47–49) to an average of 1.6 ng/g in Hy-Line White (47) and 2.13 ng/g in Bovan White (50) under control conditions. The mean concentration of corticosterone in eggs from unstressed birds has been previously reported as 1.17 in yolk and 1.55 ng/ml albumen (51). However, analytical validation of enzyme- and radio-immunoassay techniques showed the presence of cross-reactive substances that hamper the quantification of corticosterone in the yolk and albumen of eggs (52). Furthermore, recent work has shown that even when more precise techniques such as Celite or HPLC are conducted, they may not be sufficient [reviewed in (16)]. Therefore, since the exact concentration of corticosterone in eggs remains unknown, we followed the methodology proposed by Janczak et al. (32) and modified by Peixoto et al. (38), which was based on plasma corticosterone concentration of hens. Injections of 10 ng/ml cortisol diluted in sesame (CORT treatment) or sesame oil alone (Vehicle treatment) were used. In preparation for this procedure, a layer of approximately 0.5 mm of silicone sealant (General Electric, Boston, MA) was smeared on the basal tip of the shell (2 cm long × 1 cm wide) of a subsample of Control eggs 1 day before egg incubation; this sealant would help prevent gas exchange and contamination following perforation and injection through the shell. On the morning of each incubation day, Vehicle and CORT solutions were prepared. The average weight of egg content, which is estimated to be 90% of total egg weight (53), was 50, 50, and 59 g per hen age group; thus, a volume of 50 μl of either CORT or Vehicle solutions were injected into eggs from 32- to 52-week-old breeders, while 60 μl was injected into eggs from 72-week-old breeders. Injections were performed using a sterile 23-gage needle through a small hole that was perforated through the silicone layer using an egg piercer. Eggs from all treatments were immediately incubated.

## Offspring Stock: Management and Data Collection

Egg collection, incubation, and hatch occurred under similar conditions for all offspring groups. Chicks from each maternal age were individually wing-banded at hatch. The placement of chicks from each strain and treatment was randomized across rooms 40 pens equally distributed in four rooms (see **Supplementary Table 1** and **Supplementary Figures 2–4**). Each pen (3.72 $m^2$) was enriched with a perch (length: 155 cm) and litter floor. Each replicate in time aimed to comprise two pens with 20 birds each (10 female: 10 male) per treatment and strain; however, final densities varied due to lower hatchability of injected eggs (38). The test orders for the procedures described below were balanced across the period of the day for all flocks, strains, and treatments to minimize the effects of time and circadian rhythm on the results.

## Offspring Stock: Social Isolation (SI)

The social separation of young chicks from their conspecifics produces an increase in distress vocalizations and stress-induced analgesia (54), allowing for the measurement of anxiety-related behaviors. Following the methodology proposed by Sufka et al. (55), chicks between 5 and 10 days of age ($N = 701$; **Table 1**) were individually placed into a squared soundproof box (63.5 cm high × 63.5 cm deep × 63.5 cm wide) where their vocalizations were recorded. The box was constructed of solid panels, covered with acoustic fabric, and equipped with five LED lights and a microphone taped on the ceiling for recordings (**Figures 3A,B**). SI lasted 5 min and was conducted from 08:00 to 12:00 h and from 14:00 to 18:00 h in a quiet room nearby the chicks' home pen. Distress calls were recorded, saved as an MPEG-4 file using the Voice Memos application (Apple, Cupertino, USA). The total number of distress calls emitted by the chicks were counted

**TABLE 1 |** Number of chickens tested by treatment, strain, and maternal age (weeks) in the social isolation and the tonic immobility tests.

| | | Strain | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Brown 1 | | | Brown 2 | | | White 1 | | | White 2 | | | W.Leghorn | | | |
| | | Maternal age | | | Maternal age | | | Maternal age | | | Maternal age | | | Maternal age | | | Total |
| | **Treatment** | **32** | **52** | **72** | **32** | **52** | **72** | **32** | **52** | **72** | **32** | **52** | **72** | **32** | **52** | **72** | |
| **Social isolation** | Control | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 11 | 12 | 12 | 12 | 12 | 12 | 12 | **179** |
| | Maternal stress | 12 | 12 | 10 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 11 | 12 | 12 | 12 | **177** |
| | Vehicle | 12 | 12 | 11 | 10 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 11 | 12 | 12 | **176** |
| | CORT | 12 | 12 | 11 | 10 | 12 | 10 | 12 | 12 | 12 | 12 | 12 | 11 | 10 | 12 | 9 | **169** |
| **Total** | | **48** | **48** | **44** | **44** | **48** | **46** | **48** | **48** | **47** | **48** | **48** | **46** | **45** | **48** | **45** | **701** |
| **Tonic immobility** | Control | - | 11 | 12 | - | 12 | 12 | - | 13 | 12 | - | 12 | 12 | - | 10 | 12 | **118** |
| | Maternal Stress | - | 12 | 12 | - | 12 | 12 | - | 11 | 12 | - | 12 | 12 | - | 11 | 12 | **118** |
| | Vehicle | - | 12 | 11 | - | 12 | 12 | - | 12 | 12 | - | 12 | 11 | - | 12 | 12 | **118** |
| | CORT | - | 12 | 12 | - | 12 | 12 | - | 12 | 12 | - | 12 | 12 | - | 9 | 7 | **112** |
| **Total** | | **-** | **47** | **47** | **-** | **48** | **48** | **-** | **48** | **48** | **-** | **48** | **47** | **-** | **42** | **43** | **466** |

*Presented in bold are the total number of chickens tested displayed by strain and maternal age (row) and treatment (column).*

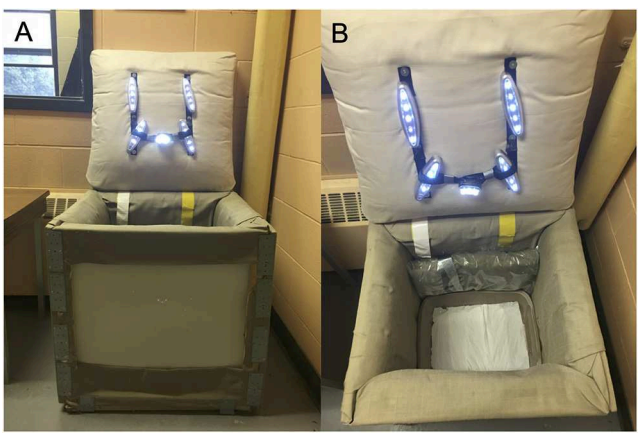


**FIGURE 3 | (A)** Soundproof box (63.5 cm high × 63.5 cm deep × 63.5 cm wide) used in the social isolation test. **(B)** The box was constructed of wood panels, covered with acoustic fabric, and equipped with five LED lights and a microphone taped on the ceiling.

by three observers blind to treatment using WavePad (NCH Software, Greenwood Village, USA).

## Offspring Stock: Tonic Immobility (TI)

A modified version of the TI methodology proposed by Jones (56) was used to measure fear in chickens at 9 weeks of age ($N = 466$; **Table 1**). Chickens were individually caught, moved into a quiet nearby room, and placed on their back in a V-shaped cradle, where the experimenter gently applied pressure on their sternum (57). If immobility lasted a minimum of 10 s, it was considered a successful induction. If not, up to three consecutive attempts at induction were performed. Each test lasted 10 min or until the bird stood up. Data were collected only from the offspring of hens of 52 and 72 weeks. Testing was conducted from 09:00 to 12:00 h and 13:00 to 16:00 h, and the procedure was recorded using a camcorder (Panasonic HC-V180K) that had been positioned perpendicularly to the cradle. Behavior was analyzed from videos and included duration until the bird rights itself up, the number of vocalizations emitted during the test, and the number of inductions needed to attain a successful induction. Data were analyzed by two trained observers blind to treatment.

Although the term TI implies in a state of reduced responsiveness that includes suppressed vocal behavior and intermittent periods of eye closure and muscle tremors in the extremities (58), different responses can be observed throughout the test (e.g., vocalization and head movement). As described by Rovee and Luciano (59), TI can be classified in three stages: In stages 1 and 2, distress calls can be emitted and eyes are either open or with occasional fluttering eyelids. Whereas, in stage 3, complete eye closure, no vocalizations, head bobbing, and occasional generalized body twitches are observed. Since these behaviors may vary in response to different methodologies [which can affect the validity of the test (57, 60)], data for the duration of stage 3 of TI, which will specifically be referred to as "3$^{rd}$ stage of TI" throughout the text, were separately recorded and analyzed.

## Statistical Analyses

The Glimmix procedure of SAS 9.4 (SAS Institute, Cary, NC) was used to perform all statistical analyses. The basic statistical model in ANOVA included fixed effects of treatment (Control, MS, Vehicle and CORT), strain (Brown 1 and 2, White 1 and 2, and White Leghorn), sex, and a treatment by strain interaction. Random effects included maternal age (32, 52, and 72 weeks) and pen (10 pens) nested in room (4 rooms), with offspring bird as the experimental unit. Further pre-planned comparisons included treatment (Control vs. MS, Control vs. Vehicle, and Control vs. CORT) and white vs. brown strains. Tests for normality included Shapiro–Wilk and Anderson Darling measurements in conjunction with visual plots. When a significant strain by treatment interaction was found, analyses controlled for the multiple testing error using the percentage of false positives, which estimates the false discovery rate [FDR (61)]. Significance was declared at $P < 0.05$. Reliability between observers (all blind

**TABLE 2 |** Average number of distress vocalizations (± SEM) performed by chicks between 5 and 10 days of age during the social isolation test.

| Treatment | Strain | | | | | Treatment average |
|---|---|---|---|---|---|---|
| | Brown 1 | Brown 2 | White 1 | White 2 | White leghorn | |
| Control | 222.3 ± 104.7$^{a,y}$ | 144.2 ± 71.0$^{a,y}$ | 119.8 ± 53.7$^{a,y}$ | 135.3 ± 61.0$^{a,y}$ | 47.7 ± 23.2$^{a,y}$ | **119.89 ± 40.2** |
| Maternal stress | 393.7 ± 178.0$^{a,y}$ | 222.1 ± 101.1$^{ab,y}$ | 60.3 ± 27.8$^{bc,y}$ | 13.8 ± 6.2$^{c,z}$ | 80.9 ± 38.3$^{ab,y}$ | **89.89 ± 30.35** |
| Vehicle | 126.0 ± 59.4$^{a,y}$ | 302.7 ± 144.0$^{a,y}$ | 60.3 ± 31.7$^{a,y}$ | 86.3 ± 39.0$^{a,y}$ | 203.7 ± 100.9$^{a,y}$ | **132.15 ± 44.9** |
| CORT | 118.7 ± 58.0$^{a,y}$ | 220.4 ± 116.3$^{a,y}$ | 63.8 ± 29.5$^{a,y}$ | 44.4 ± 21.1$^{a,yz}$ | 213.0 ± 110.6$^{a,y}$ | **109.56 ± 38.0** |
| **Strain average** | **190.05 ± 66.6$^{a}$** | **215.01 ± 76.90$^{a}$** | **72.64 ± 35.84$^{bc}$** | **51.65 ± 18.0$^{c}$** | **113.75 ± 58.0$^{ab}$** | |

*Results are presented by strain and treatment. Means in the same row with different letter superscripts (a, b, c) differ significantly between strains ($P < 0.05$). Means in the same column with different letter superscripts (y, z) differ significantly among treatments ($P < 0.05$). Presented in bold are the average number of distress vocalization per strain (row) and treatment (column).*

to treatment) was calculated using Kendall's Tau-b coefficient. Kendall's $\tau$ score of 1.0 is considered a perfect relationship, and a score of 0.7 is considered acceptable (62). Consequently, scores reported for SI (Kendall's $\tau = 0.93$; $P < 0.001$) and duration of TI (Kendall's $\tau = 0.82$; $P < 0.001$) indicate agreement among observers.

### Social Isolation

The SI data were subjected to the basic model and log-normally transformed to meet the assumption of a normal distribution of residuals. Significance post-FDR correction was set at $P < 0.005$ and followed by a power analysis (alpha = 0.005). Least square (LS-) means and standard error of means (SEM) were back-transformed and are presented in the results as the average of distress vocalizations.

### Tonic Immobility

The duration of immobility and number of vocalizations were subjected to the basic statistical model in ANOVA. To meet the assumption of a normal distribution of residuals, data for duration were subjected to a log-normal transformation, while vocalization data were transformed by the arcsine of the square root. Random effects were grouped by strain. LS-Means and standard deviation (SD) of both tests were back-transformed and are presented in the results as the average duration of TI in seconds and the average number of calls emitted during the test. The number of attempts needed for induction is presented as a percentage of birds; data were subjected to a Poisson transformation but were not normally distributed when the model included a strain by treatment interaction. Thus, a simpler statistical model containing only treatment as the fixed effect was used. Differences between LS-means were tested using a chi-square test. Due to the small number of birds induced into stage 3 of TI ($n = 41$), residuals for measurements of duration were not normally distributed when the model included a strain by treatment interaction. Therefore, a simpler statistical model containing only strain, treatment, and sex as fixed effects was used.

## RESULTS

### Social Isolation

The number of distress calls expressed by the offspring of layer breeders was affected by strain and stress treatment ($P < 0.001$; **Table 2**). Chicks of the White 2 strain vocalized less when their mothers were subjected to MS compared to Control ($P < 0.001$). Similarly, MS breeders from the Brown 1, Brown 2, and White Leghorn strains produced chicks that vocalized more than White 2. Overall, brown chicks vocalized more than white ($P < 0.001$), and sex displayed a trend toward significance ($P = 0.066$), with females (125.7 ± 39.6 calls) vocalizing more than males (99.4 ± 31.3 calls).

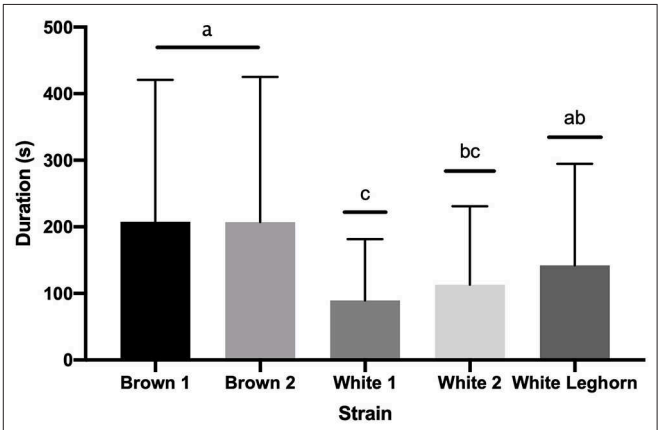


**FIGURE 4 |** Duration (s) of tonic immobility displayed by strain (+ SD). Means with different letter superscripts$^{(a-c)}$ differ ($P < 0.05$).

### Tonic Immobility

The duration of TI in 9-week-old offspring of layer breeders was not affected by an interaction of strain by treatment ($P = 0.105$), treatment ($P = 0.924$), or sex ($P = 0.643$); but brown chickens stayed longer ($P < 0.001$) in TI than white (**Figure 4**). The duration of the third stage of TI was not affected by treatment ($P = 0.863$), strain ($P = 0.701$), or sex ($P = 0.089$).

The number of vocalizations expressed by offspring in TI was also not affected by an interaction of strain by treatment ($P = 0.580$) or treatment ($P = 0.325$). However, chickens of brown strain vocalized more ($P < 0.003$) than white (**Figure 5**), and pullets (10.2 ± 1.2 calls) vocalized more ($P < 0.001$) than cockerels (3.05 ± 0.7 calls).

The number of attempts needed to induce a chicken into TI was not affected by treatment ($P = 0.892$). More chickens from the Brown 1 strain needed a second attempt to reach TI compared to the White 1 strain ($P = 0.015$) (**Figure 6**).

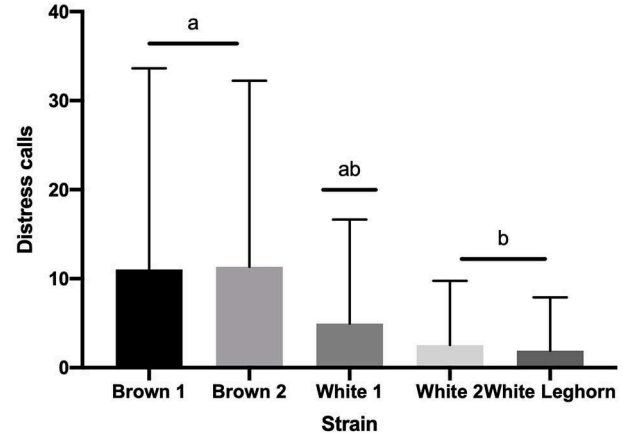


**FIGURE 5 |** Number of distress calls (+SD) emitted during tonic immobility displayed by strain. Means with different letter superscripts[a,b] differ ($P < 0.05$).

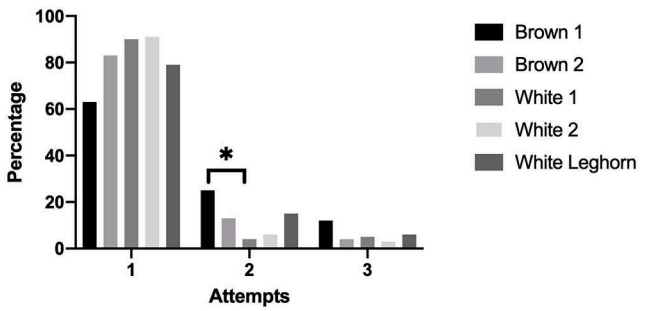


**FIGURE 6 |** Percentage of chickens that were induced into tonic immobility after 1, 2, or 3 attempts displayed by strain (*$P < 0.05$).

# DISCUSSION

## Limitations and Effects of the Stress Treatments

This study aimed to determine the effects of maternal stress on the behavior of different strains of laying hens. We hypothesized that the CORT treatment would show a clear response acting as a positive control treatment, while MS would highlight genetic differences among strains. In contrast to our hypothesis, the CORT treatment showed no effects on the behavior of the offspring and MS decreased the number of distress calls expressed by the offspring of White 2 mothers during SI but showed no differences in TI.

One limitation of this study is that the acute stressors used in the MS treatment were based on reports in the literature and not validated in our population of layer breeders, with the exception of the physical restraint test. The HPA axis activation of a subsample of layer breeders from all strains and treatment groups was tested at 75 weeks of age ($N = 119$). Breeders from both MS and Control treatments produced elevated concentrations of corticosterone in response to the restraint test [baseline control: 2.37 ± 0.49 ng/ml; baseline MS: 2.97 ± 0.47 ng/ml ($P = 0.822$); stress response control: 5.24 ± 0.55; stress response MS: 5.73 ± 0.55 ($P = 0.841$)] confirming that layers from the MS treatment were still physiologically responsive to restraint after repeated exposure (unpublished data). Nevertheless, we were unable to measure if this transient increase in plasma corticosterone was enough to alter the egg composition. Lastly, corticosterone has a short lifetime in chickens [∼22 min (63)], and each stressor used in the study lasted a maximum of 30 min from catching until layers were returned to their home pen. Chronic stress is likely more important to signal the offspring than the short-term, acute stressors used in our experiment.

Once viewed as a successful model for testing the effects of maternal stress (6), the largely unnatural and invasive aspects of the egg injection methodology should be carefully considered. Firstly, the actual concentration of corticosterone transferred from mother to egg remains unknown (52, 64), may differ across strains (47), and can potentially overwhelm the embryo if outside of the physiological range of eggs. Indeed, as published in Peixoto et al. (38), the average hatchability for the control treatment of this study was 83%, whereas hatchability for the vehicle and control treatments were 38 and 25%, respectively. The decrease in hatchability in the vehicle treatment suggests that mechanical damage such as puncturing and disrupting eggshell membranes (which might increase the chances of pushing eggshell particles into the albumen) or the chemical composition of the vehicle affected the progeny. It is also possible that the silicone layer used to seal the hole was applied ineffectively, leaving an open hole in the shell that facilitated contamination. Lastly, the high levels of embryonic mortality in the injected groups may have created a subset of birds that were more resistant to the adverse effects of the injection, limiting the generalization of the results presented herein. Until a precise method for quantifying corticosterone in the egg and less invasive procedures are available, the efficacy of this methodology and the biological relevance of the corticosterone dosage used in the present experiment are debatable.

SI is a well-validated test that has been used as an *in vivo* preclinical screening of anxiolytic drugs (65, 66), which were shown to reverse distress vocalizations and pain-related behavior in chicks (67). Moreover, birds tested with and without the presence of a mirror confirmed the assumption that vocalizations increased due to an absence of conspecifics (68). In the present study, the offspring of the White 2 MS breeders vocalized less than the Control treatment of the same strain. To our knowledge, this is the first study that evaluated the effects of maternal stress on anxiety-like behavior through the SI test, and the results are congruent with those observed in quails tested in an open field test (14). Interestingly, the eggs from stressed quails showed higher concentrations of testosterone compared to control groups. Androgenic hormones such as testosterone are known to be important mediators of maternal effects on the behavior of the offspring (69, 70), possibly more than corticosterone [reviewed in (16, 71, 72)]. In addition, genetic differences across strains display a higher susceptibility of the White 2 strain to maternal stress compared to the other strains used in this study. Nevertheless, only minimal outcomes were observed in the progeny of the MS breeders, suggesting a higher resiliency to stressors than expected.

Tonic immobility is a state of reduced responsiveness thought to be a defense strategy used to decrease the predator's interest in the prey (73). It is induced by physical restraint, and its duration is considered a measure of fearfulness in birds (56, 57). Our lack of treatment effects in TI corroborates with Rubolini et al. (26), who injected corticosterone into fertile eggs of yellow-legged gulls. Contrary to our findings, the offspring of hens subjected to an unpredictable feeding schedule stayed longer in TI (13). This stressor, however, is not necessarily associated with increased levels of corticosterone in the egg and may be translated to the offspring via different pathways (e.g., nutrition). Also using a single egg injection of corticosterone prior to incubation, Janczak et al. (74) observed that chicks from injected eggs stayed longer in TI but only if they had been previously handled, suggesting that life experiences influence this behavioral effect of maternal stress. Interestingly, physiological studies on maternal stress and the HPA axis activation of the offspring showed that treatment effects are only observed when the offspring is also subjected to stressors (75–77). Therefore, a combination of maternal stress and life experience might be essential to trigger behavioral and physiological responses in the offspring. Our lack of treatment effects in TI might, thus, be related to a natural preservation of the phenotype of the offspring, since behavioral changes can easily become detrimental. This has important consequences for predicting and managing maternal effects in both breeder and commercial flocks, which may be regularly exposed to stressful events.

Analyses of the duration of the 3rd stage TI failed to display any effects of treatment or strain. Although the description of a bird in the 3rd stage (i.e., complete eye closure, no vocalizations, head bobbing, and occasional generalized body twitches) seems more similar to the original description of TI by Nash et al. (58), it is possible that the rigorousness of the method (which excludes birds with their eyes open and vocalizing, common behaviors during TI) may have reduced the test's ability to detect subtle behavioral differences, and therefore, it is not recommended.

## Effect of Strain

Strain effects were found in both behavior tests. Contrast statements showed that the differences were primarily associated with the phylogenetically distant brown and white strains. The brown strains vocalized more in SI and TI and showed longer durations of immobility during TI, suggesting a higher occurrence of anxious and fearful behaviors compared to the white lines. This variability might be due to the intense genetic selection for productive traits in the domestic layer or by the phylogenetic, behavioral, and physiological differences across strains (34, 36, 47, 78, 79), which might be explained by evolution and domestication. Population studies exploring genetic diversity showed that brown lines originally came from African and Mediterranean genetic clustering, whereas white lines originated from the European cluster [reviewed by (78)]. Moreover, commercial brown lines are based from the Rhode Island Red, an originally dual-purpose breed (selected for both meat and eggs) with medium genetic diversity, whereas commercial white lines are based from White Leghorn, a low genetic diversity breed (80).

Genetic selection for production traits may have also affected the behavior of chickens if the traits are correlated or genetically linked. Several quantitative trait loci (QTL) related to fear response, for example, have been found on different chromosomes in White Leghorns. More specifically, TI was associated with three different QTLs on chromosome 1 that coincide with the position of two major QTLs for growth and bodyweight (81, 82). Therefore, genetic selection for body weight may have simultaneously affected fearfulness in White Leghorn. However, data for these studies were obtained exclusively from one strain, and it would be important to measure if this is also valid for lines expressing different genetics, such as brown strains. An early study of genetic differences and behavior showed that White Leghorns chicks displayed longer duration of TI than a Production Red strain, and when the two strains were crossbred, offspring showed intermediate durations (83), supporting the hypothesis that behavioral differences between brown and white strains are genetically dependent.

Contrary to our findings on vocalization (in both SI and TI) and duration of TI, the measurement of the number of attempts to attain a successful induction in TI showed that Brown 1 needed more second attempts than White 1, therefore suggesting that for this particular trait, a brown strain was less fearful than a white strain. Overall, results on anxiety and fearfulness found in the literature are often inconsistent, and a bird's motivation to engage in certain behaviors remains unclear. For example, some studies have found that brown strains lasted longer in TI (35, 36, 84, 85) and vocalized less than white strains in an open field test (86). The interpretation of these tests is thus difficult, with a multitude of factors such as genetic selection (87), hormones (88), the environment (89), and test methodology simultaneously affecting the behavior of layers.

## Effect of Sex

In accordance with previous research (25, 41, 90), the present study did not show an interaction between treatment and sex to affect measures of anxiety and fearfulness in the offspring. Nevertheless, the current study suggests that female chickens are more anxious than males, displaying a higher frequency of distress calls during TI and a similar trend pattern in SI. These findings corroborate with Jones (86), who observed that hens were more active and vocalized more than cockerels in an open field test.

The development of sexual dimorphism in behavior is mostly related to the influence of gonadal hormones, androgens, and estrogens on the nervous system (91). Individually and combined, these hormones can organize and reorganize the neuronal circuitry involved in neuroendocrine and behavioral functions, including the serotonin system (91, 92) that is responsible for anxiety traits (93, 94). Moreover, the environment can also interact with sex to affect behavior. For example, Vallorttgara and Zanforlin (95) found that social isolation from cage companions was more stressful for female than male chickens. In the current study, birds were separated from conspecifics at both tests. Consequently, the hens might have vocalized more due to an intensified emotional response experienced during the tests.

## CONCLUSION

Our findings suggest that the effects of maternal stress on measures of anxiety and fearfulness were contingent on genetic strain, but only when stressors are applied directly to the mother. The lack of CORT treatment effect suggests that maternal stress may not be mediated by corticosterone. Additionally, genetic strains responded differently to both behavior tests, with brown birds displaying higher levels of fearfulness in comparison to white strains, suggesting genetic differences in fear behavior across the genetic lines of commercial layers. These findings have important implications, since behavioral variations can be decisive to determine the overall adaptability of a strain to a specific production system. Moreover, in research settings, researchers must take into consideration behavioral differences when assessing different strains of laying hens, since generalization might be misleading.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of Guelph Animal Care Committee (Animal Utilization Protocol #1946).

## AUTHOR CONTRIBUTIONS

TW conceived the work and prepared the grants. TW and MP designed the study and prepared the manuscript. NK and AN contributed to the conception of the study. MP conducted the work and analyzed the data. All authors reviewed and approved the final manuscript.

## FUNDING

This research was funded by Egg Farmers of Canada, the Ontario Ministry of Agriculture and Rural Affairs (OMAFRA), the Canadian Poultry Research Council, Hendrix Genetics, and Lohmann Tierzucht.

## ACKNOWLEDGMENTS

We thank Dr. Andrew Janczak for his comments and suggestions; Daniel Rothschild, Priyanka Thavarajah, and Madeleine Browne for the assistance with data collection; and the personnel at the University of Guelph's Arkell Poultry Research Station for the technical assistance and maintenance of the birds.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00128/full#supplementary-material

## REFERENCES

1. Weinstock M. The long-term behavioural consequences of prenatal stress. *Neurosci Biobehav Rev*. (2008) 32:1073–86. doi: 10.1016/j.neubiorev.2008.03.002
2. Thompson WR. Influence of prenatal maternal anxiety on emotionality in young rats. *Science*. (1957) 126:73–4. doi: 10.1126/science.126.3263.73-a
3. Painter RC, Roseboom TJ, de Rooij SR. Long-term effects of prenatal stress and glucocorticoid exposure. *Birth Defects Res Part C Embryo Today*. (2012) 96:315–24. doi: 10.1002/bdrc.21021
4. Kapoor A, Matthews SG. Prenatal stress modifies behavior and hypothalamic-pituitary-adrenal function in female guinea pig offspring: effects of timing of prenatal stress and stage of reproductive cycle. *Endocrinology*. (2008) 149:6406–15. doi: 10.1210/en.2008-0347
5. Pinson SE, Wilson JL, Navara KJ. Timing matters: corticosterone injections 4 h before ovulation bias sex ratios towards females in chickens. *J Comp Physiol B*. (2015) 185:539–46. doi: 10.1007/s00360-015-0897-5
6. Henriksen R, Rettenbacher S, Groothuis TGG. Prenatal stress in birds: pathways, effects, function and perspectives. *Neurosci Biobehav Rev*. (2011) 35:1484–501. doi: 10.1016/j.neubiorev.2011.04.010
7. Dixon LM, Sparks NHC, Rutherford KMD. Early experiences matter: a review of the effects of prenatal environment on offspring characteristics in poultry. *Poult Sci*. (2016) 95:489–99. doi: 10.3382/ps/pev343
8. Kapoor A, Petropoulos S, Matthews SG. Fetal programming of hypothalamic-pituitary-adrenal (HPA) axis function and behavior by synthetic glucocorticoids. *Brain Res Rev*. (2008) 57:586–95. doi: 10.1016/j.brainresrev.2007.06.013
9. Belliure J, Meylan S, Clobert J. Prenatal and postnatal effects of corticosterone on behavior in juveniles of the common lizard, *Lacerta vivipara*. *J Exp Zool A Comp Exp Biol*. (2004) 301:401–10. doi: 10.1002/jez.a.20066
10. Nätt D, Lindqvist N, Stranneheim H, Lundeberg J, Torjesen PA, Jensen P. Inheritance of acquired behaviour adaptations and brain gene expression in chickens. *PLoS ONE*. (2009) 4:e6405. doi: 10.1371/annotation/4f90ac09-ae5e-469a-a2f3-21a5ac68dc31
11. Davis M. The role of the amygdala in fear-potentiated startle: implications for animal models of anxiety. *Trends Pharmacol Sci*. (1992) 13:35–41. doi: 10.1016/0165-6147(92)90014-W
12. Dalley JW, Cardinal RN, Robbins TW. Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. *Neurosci Biobehav Rev*. (2004) 28:771–84. doi: 10.1016/j.neubiorev.2004.09.006
13. Janczak AM, Torjesen P, Palme R, Bakken M. Effects of stress in hens on the behaviour of their offspring. *Appl Anim Behav Sci*. (2007) 107:66–77. doi: 10.1016/j.applanim.2006.09.016
14. Guibert F, Richard-Yris MA, Lumineau S, Kotrschal K, Bertin A, Petton C, et al. Unpredictable mild stressors on laying females influence the composition of Japanese quail eggs and offspring's phenotype. *Appl Anim Behav Sci*. (2011) 132:51–60. doi: 10.1016/j.applanim.2011.03.012
15. Cottrell EC, Seckl JR. Prenatal stress, glucocorticoids and the programming of adult disease. *Front Behav Neurosci*. (2009) 3:19. doi: 10.3389/neuro.08.019.2009
16. Groothuis TGG, Hsu B-Y, Kumar N, Tschirren B. Revisiting mechanisms and functions of prenatal hormone-mediated maternal effects using avian species as a model. *Philos Trans R Soc B Biol Sci*. (2019) 374:20180115. doi: 10.1098/rstb.2018.0115
17. Ruuskanen S, Hsu B-Y. Maternal thyroid hormones: an unexplored mechanism underlying maternal effects in an ecological framework. *Physiol Biochem Zool*. (2018) 91:904–16. doi: 10.1086/697380
18. Possenti CD, Secomandi S, Schiavon A, Caprioli M, Rubolini D, Romano A, et al. Independent and combined effects of egg pro- and anti-oxidants on gull chick phenotype. *J Exp Biol*. (2018) 221:jeb174300. doi: 10.1242/jeb.174300

19. Roth O, Beemelmanns A, Barribeau SM, Sadd BM. Recent advances in vertebrate and invertebrate transgenerational immunity in the light of ecology and evolution. *Heredity*. (2018) 121:225–38. doi: 10.1038/s41437-018-0101-2
20. MacDougall-Shackleton SA, Bonier F, Romero LM, Moore IT. Glucocorticoids and "Stress" are not synonymous. *Integr Org Biol*. (2019) 1:obz017. doi: 10.1093/iob/obz017
21. Ahmed AA, Musa HH, Sifaldin AZ. Prenatal corticosterone exposure programs growth, behavior, reproductive function and genes in the chicken. *Asian Pac J Reprod*. (2016) 5:271–8. doi: 10.1016/j.apjr.2016.06.013
22. Jenkins SA, Porter TE. Ontogeny of the hypothalamo-pituitary-adrenocortical axis in the chicken embryo: a review. *Domest Anim Endocrinol*. (2004) 26:267–75. doi: 10.1016/j.domaniend.2004.01.001
23. Garamszegi LZ, Rosivall B, Rettenbacher S, Markó G, Zsebok S, Szöllosi E, et al. Corticosterone, avoidance of novelty, risk-taking and aggression in a wild bird: no evidence for pleiotropic effects. *Ethology*. (2012) 118:621–35. doi: 10.1111/j.1439-0310.2012.02049.x
24. Janczak AM, Braastad BO, Bakken M. Behavioural effects of embryonic exposure to corticosterone in chickens. *Appl Anim Behav Sci*. (2006) 96:69–82. doi: 10.1016/j.applanim.2005.04.020
25. Henriksen R, Rettenbacher S, G.G. Groothuis T. Maternal corticosterone elevation during egg formation in chickens (*Gallus gallus domesticus*) influences offspring traits, partly via prenatal undernutrition. *Gen Comp Endocrinol*. (2013) 191:83–91. doi: 10.1016/j.ygcen.2013.05.028
26. Rubolini D, Romano M, Boncoraglio G, Ferrari RP, Martinelli R, Galeotti P, et al. Effects of elevated egg corticosterone levels on behavior, growth, and immunity of yellow-legged gull (*Larus michahellis*) chicks. *Horm Behav*. (2005) 47:592–605. doi: 10.1016/j.yhbeh.2005.01.006
27. Rodricks CL, Miller SL, Jenkin G, Gibbs ME. The role of corticosterone in prehatch-induced memory deficits in chicks. *Brain Res*. (2006) 1123:34–41. doi: 10.1016/j.brainres.2006.09.028
28. Lay DC, Wilson ME. Development of the chicken as a model for prenatal stress. *J Anim Sci*. (2002) 80:1954–61. doi: 10.2527/2002.8071954x
29. Henriksen R, Groothuis TG, Rettenbacher S. Elevated plasma corticosterone decreases yolk testosterone and progesterone in chickens: linking maternal stress and hormone-mediated maternal effects. *PLoS ONE*. (2011) 6:e23824. doi: 10.1371/journal.pone.0023824
30. von Engelhardt N, Henriksen R, Groothuis TGG. Steroids in chicken egg yolk: metabolism and uptake during early embryonic development. *Gen Comp Endocrinol*. (2009) 163:175–83. doi: 10.1016/j.ygcen.2009.04.004
31. Heiblum R, Arnon E, Chazan G, Robinzon B, Gvaryahu G, Snapir N. Glucocorticoid administration during incubation: embryo mortality and posthatch growth in chickens. *Poult Sci*. (2001) 80:1357–63. doi: 10.1093/ps/80.9.1357
32. Janczak AM, Haung A, Bakken M. Evaluation of experimental methods for manipulating chicken egg hormone content using injections. *J Anim Vet Adv*. (2007) 6:500–4.
33. Rettenbacher S, Möstl E, Groothuis TGG. Gestagens and glucocorticoids in chicken eggs. *Gen Comp Endocrinol*. (2009) 164:125–9. doi: 10.1016/j.ygcen.2009.05.019
34. de Haas EN, Kemp B, Bolhuis JE, Groothuis T, Rodenburg TB. Fear, stress, and feather pecking in commercial white and brown laying hen parent-stock flocks and their relationships with production parameters. *Poult Sci*. (2013) 92:2259–69. doi: 10.3382/ps.2012-02996
35. Fraisse F, Cockrem JF. Corticosterone and fear behaviour in white and brown caged laying hens. *Br Poult Sci*. (2006) 47:110–9. doi: 10.1080/00071660600610534
36. Pusch EA, Bentz AB, Becker DJ, Navara KJ. Behavioral phenotype predicts physiological responses to chronic stress in proactive and reactive birds. *Gen Comp Endocrinol*. (2018) 255:71–7. doi: 10.1016/j.ygcen.2017.10.008
37. Nätt D, Nätt D, Rubin C-J, Wright D, Johnsson M, Beltéky J, et al. Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. *BMC Genomics*. (2012) 13:59. doi: 10.1186/1471-2164-13-59
38. Peixoto MRLV, Karrow NA, Widowski TM. Effects of prenatal stress and genetics on embryonic survival and offspring growth of laying hens. *Poult Sci*. (2020) 99:1618–27. doi: 10.1016/j.psj.2019.10.018
39. Nielsen H. Hen age and fatty acid composition of egg yolk lipid. *Br Poult Sci*. (1998) 39:53–6. doi: 10.1080/00071669889394
40. Beuving G, Vonder GMA. Effect of stressing factors on corticosterone levels in the plasma of laying hens. *Gen Comp Endocrinol*. (1978) 35:153–9. doi: 10.1016/0016-6480(78)90157-0
41. Ericsson M, Henriksen R, Bélteky J, Sundman AS, Shionoya K, Jensen P. Long-term and transgenerational effects of stress experienced during different life phases in chickens (*Gallus gallus*). *PLoS ONE*. (2016) 11:e0153879. doi: 10.1371/journal.pone.0153879
42. Pitk M, Tilgar V, Kilgas P, Mänd R. Acute stress affects the corticosterone level in bird eggs: a case study with great tits (*Parus major*). *Horm Behav*. (2012) 62:475–9. doi: 10.1016/j.yhbeh.2012.08.004
43. Rich EL, Romero LM. Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am J Physiol Regul Integr Comp Physiol*. (2005) 288:R1628–36. doi: 10.1152/ajpregu.00484.2004
44. Siegel HS. Physiological stress in birds. *Bioscience*. (1980) 30:529–34. doi: 10.2307/1307973
45. Scanes CG. Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio. *Poult Sci*. (2016) 95:2208–15. doi: 10.3382/ps/pew137
46. Johnston PA, Liu H, O'Connell T, Phelps P, Bland M, Tyczkowski J, et al. Applications in in ovo technology. *Poult Sci*. (1997) 76:165–78. doi: 10.1093/ps/76.1.165
47. Navara KJ, Pinson SE. Yolk and albumen corticosterone concentrations in eggs laid by white versus brown caged laying hens. *Poult Sci*. (2010) 89:1509–13. doi: 10.3382/ps.2009-00416
48. Ahmed AA, Ma W, Ni Y, Wang S, Zhao R. Corticosterone in ovo modifies aggressive behaviors and reproductive performances through alterations of the hypothalamic-pituitary-gonadal axis in the chicken. *Anim Reprod Sci*. (2014) 146:193–201. doi: 10.1016/j.anireprosci.2014.02.013
49. Engel JM, Widowski TM, Tilbrook AJ, Butler KL, Hemsworth PH. The effects of floor space and nest box access on the physiology and behavior of caged laying hens. *Poult Sci*. (2019) 98:533–47. doi: 10.3382/ps/pey378
50. Haussmann MF, Longenecker AS, Marchetto NM, Juliano SA, Bowden RM. Embryonic exposure to corticosterone modifies the juvenile stress response, oxidative stress and telomere length. *Proc R Soc B Biol Sci*. (2012) 279:1447–56. doi: 10.1098/rspb.2011.1913
51. Eriksen MS, Haug A, Torjesen PA, Bakken M. Prenatal exposure to corticosterone impairs embryonic development and increases fluctuating asymmetry in chickens (*Gallus gallus domesticus*). *Br Poult Sci*. (2003) 44:690–7. doi: 10.1080/00071660310001643660
52. Rettenbacher S, Groothuis TG, Henriksen R, Möstl E. Corticosterone in bird eggs: the importance of analytical validation. *Wien Tierarztl Monatsschr*. (2013) 100:283–90.
53. Beuving G, Vonder GMA. The influence of ovulation and oviposition on corticosterone levels in the plasma of laying hens. *Gen Comp Endocrinol*. (1981) 44:382–8. doi: 10.1016/0016-6480(81)90016-2
54. Sufka KJ, Weed NC. Construct validation of behavioral indices of isolation stress and inflammatory nociception in young domestic fowl. *Physiol Behav*. (1994) 55:741–6. doi: 10.1016/0031-9384(94)90054-X
55. Sufka KJ, Feltenstein MW, Warnick JE, Acevedo EO, Webb HE, Cartwright CM. Modeling the anxiety-depression continuum hypothesis in domestic fowl chicks. *Behav Pharmacol*. (2006) 17:681–9. doi: 10.1097/FBP.0b013e3280115fac
56. Jones RB. The tonic immobility reaction of the domestic fowl: a review. *Worlds Poult Sci J*. (1986) 42:82–96. doi: 10.1079/WPS19860008
57. Forkman B, Boissy A, Meunier-Salaün MC, Canali E, Jones RB. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. *Physiol Behav*. (2007) 91:531–65. doi: 10.1016/j.physbeh.2007.03.016
58. Nash RF, Gallup GG, Czech DA. Psychophysiological correlates of tonic immobility in the domestic chicken (*Gallus gallus*). *Physiol Behav*. (1976) 17:413–8. doi: 10.1016/0031-9384(76)90100-1
59. Rovee CK, Luciano DP. Rearing influences on tonic immobility in three-day-old chicks (*Gallus gallus*). *J Comp Physiol Psychol*. (1973) 83:351–4. doi: 10.1037/h0034429
60. Jones RB, Faure JM. Sex and strain comparisons of tonic immobility ("Righting time") in the domestic fowl and the effects

of various methods of induction. *Behav Processes*. (1981) 6:47–55. doi: 10.1016/0376-6357(81)90015-2
61. Garcia LV. Controlling the false discovery rate in ecological research. *Trends Ecol Evol*. (2016) 18:553–4. doi: 10.1016/j.tree.2003.08.011
62. Arndt S, Turvey C, Andreasen NC. Correlating and predicting psychiatric symptom ratings: Spearman's v versus Kendall's tau correlation. *J Psychiatr Res*. (1999) doi: 10.1016/S0022-3956(98)90046-2
63. Birrenkott GP, Wiggins ME. Determination of dexamethasone and corticosterone half-lives in male broilers. *Poult Sci*. (1984) 63:1064–8. doi: 10.3382/ps.0631064
64. Almasi B, Rettenbacher S, Müller C, Brill S, Wagner H, Jenni L. Maternal corticosterone is transferred into the egg yolk. *Gen Comp Endocrinol*. (2012) 178:139–44. doi: 10.1016/j.ygcen.2012.04.032
65. Smith KK, Dharmaratne HRW, Feltenstein MW, Broom SL, Roach JT, Nanayakkara NPD, et al. Anxiolytic effects of kava extract and kavalactones in the chick social separation-stress paradigm. *Psychopharmacology*. (2001) 155:86–90. doi: 10.1007/s002130100686
66. Sufka KJ, Roach JT, Chambliss WG, Broom SL, Feltenstein MW, Wyandt CM, et al. Anxiolytic properties of botanical extracts in the chick social separation-stress procedure. *Psychopharmacology*. (2001) 153:219–24. doi: 10.1007/s002130000571
67. Watson GS, Sufka KJ. Chlordiazepoxide reverses social-separation-induced distress vocalizations and analgesia in young domestic fowl. *Exp Clin Psychopharmacol*. (1996) 4:347–53. doi: 10.1037/1064-1297.4.4.347
68. Feltenstein MW, Lambdin LC, Webb HE, Warnick JE, Khan SI, Khan IA, et al. Corticosterone response in the chick separation-stress paradigm. *Physiol Behav*. (2003) 78:489–93. doi: 10.1016/S0031-9384(03)00030-1
69. Guibert F, Richard-Yris MA, Lumineau S, Kotrschal K, Möstl E, Houdelier C. Yolk testosterone levels and offspring phenotype correlate with parental age in a precocial bird. *Physiol Behav*. (2012) 105:242–50. doi: 10.1016/j.physbeh.2011.08.009
70. Niall Daisley J, Bromundt V, Möstl E, Kotrschal K. Enhanced yolk testosterone influences behavioral phenotype independent of sex in Japanese quail chicks *Coturnix japonica*. *Horm Behav*. (2005) 47:185–94. doi: 10.1016/j.yhbeh.2004.09.006
71. Groothuis TGG, Schwabl H. Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? *Philos Trans R Soc B Biol Sci*. (2008) 363:1647–61. doi: 10.1098/rstb.2007.0007
72. Groothuis TGG, Müller W, von Engelhardt N, Carere C, Eising C. Maternal hormones as a tool to adjust offspring phenotype in Avian species. *Neurosci Biobehav Rev*. (2005) 29:329–52. doi: 10.1016/j.neubiorev.2004.12.002
73. Thompson RKR, Foltin RW, Boylan RJ, Sweet A, Graves CA, Lowitz CE. Tonic immobility in Japanese quail can reduce the probability of sustained attack by cats. *Anim Learn Behav*. (1981) 9:145–9. doi: 10.3758/BF03212037
74. Janczak AM, Heikkilä M, Valros A, Torjesen P, Andersen IL, Bakken M. Effects of embryonic corticosterone exposure and post-hatch handling on tonic immobility and willingness to compete in chicks. *Appl Anim Behav Sci*. (2007) 107:275–86. doi: 10.1016/j.applanim.2006.10.002
75. Carter AW, Bowden RM, Paitz RT. Evidence of embryonic regulation of maternally derived yolk corticosterone. *J Exp Biol*. (2018) 221:jeb.182600. doi: 10.1242/jeb.182600
76. Vassallo BG, Paitz RT, Fasanello VJ, Haussmann MF. Glucocorticoid metabolism in the in ovo environment modulates exposure to maternal corticosterone in Japanese quail embryos (*Coturnix japonica*). *Biol Lett*. (2014) 10:20140502. doi: 10.1098/rsbl.2014.0502
77. Vassallo BG, Litwa HP, Haussmann MF, Paitz RT. In ovo metabolism and yolk glucocorticoid concentration interact to influence embryonic glucocorticoid exposure patterns. *Gen Comp Endocrinol*. (2019) 272:57–62. doi: 10.1016/j.ygcen.2018.11.013
78. Tixier-Boichard M, Bed'Hom B, Rognon X. Chicken domestication: from archeology to genomics. *C R Biol*. (2011) 334:197–204. doi: 10.1016/j.crvi.2010.12.012
79. Groothuis TGG, Carere C. Avian personalities: characterization and epigenesis. *Neurosci Biobehav Rev*. (2005) 29:137–50. doi: 10.1016/j.neubiorev.2004.06.010
80. Lyimo CM, Weigend A, Msoffe PL, Eding H, Simianer H, Weigend S. Global diversity and genetic contributions of chicken populations from African, Asian and European regions. *Anim Genet*. (2014) 45:836–48. doi: 10.1111/age.12230
81. Schütz KE, Kerje S, Jacobsson L, Forkman B, Carlborg Ö, Andersson L, et al. Major growth QTLs in fowl are related to fearful behavior: possible genetic links between fear responses and production traits in a red junglefowl x white leghorn intercross. *Behav Genet*. (2004) 34:121–30. doi: 10.1023/B:BEGE.0000009481.98336.fc
82. Kerje S, Carlborg Ö, Jacobsson L, Schütz K, Hartmann C, Jensen P, et al. The twofold difference in adult size between the red junglefowl and white leghorn chickens is largely explained by a limited number of QTLs. *Anim Genet*. (2003) 34:264–74. doi: 10.1046/j.1365-2052.2003.01000.x
83. Gallup GG, Ledbetter DH, Maser JD. Strain differences among chickens in tonic immobility: evidence for an emotionality component. *J Comp Physiol Psychol*. (1976) 90:1075–81. doi: 10.1037/h0078662
84. Albentosa MJ, Kjaer JB, Nicol CJ. Strain and age differences in behaviour, fear response and pecking tendency in laying hens. *Br Poult Sci*. (2003) 44:333–44. doi: 10.1080/00071660310001598085
85. Mahboub HDH, Müller J, von Borell E. Outdoor use, tonic immobility, heterophil/lymphocyte ratio and feather condition in free-range laying hens of different genotype. *Br Poult Sci*. (2004) 45:738–44. doi: 10.1080/00071660400014267
86. Jones R. Sex and strain differences in the open-field responses of the domestic chick. *Appl Anim Ethol*. (1977) 3:255–61. doi: 10.1016/0304-3762(77)90006-2
87. Dennis RL, Chen ZQ, Cheng HW. Serotonergic mediation of aggression in high and low aggressive chicken strains. *Poult Sci*. (2008) 87:612–20. doi: 10.3382/ps.2007-00389
88. Cockrem JF. Stress, corticosterone responses and avian personalities. *J Ornithol*. (2007) 148:169–78. doi: 10.1007/s10336-007-0175-8
89. Uitdehaag K, Komen H, Rodenburg TB, Kemp B, van Arendonk J. The novel object test as predictor of feather damage in cage-housed Rhode Island red and white leghorn laying hens. *Appl Anim Behav Sci*. (2008) 109:292–305. doi: 10.1016/j.applanim.2007.03.008
90. Goerlich VC, Nätt D, Elfwing M, Macdonald B, Jensen P. Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken. *Horm Behav*. (2012) 61:711–8. doi: 10.1016/j.yhbeh.2012.03.006
91. Palanza P. Animal models of anxiety and depression: how are females different? *Neurosci Biobehav Rev*. (2001) 25:219–33. doi: 10.1016/S0149-7634(01)00010-0
92. Carlsson M, Carlsson A. A regional study of sex differences in rat brain serotonin. *Prog Neuropsychopharmacol Biol Psychiatry*. (1988) 12:53–61. doi: 10.1016/0278-5846(88)90061-9
93. Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, et al. Elevated anxiety and antidepressant-like responses in serotonin 5-HT(1A) receptor mutant mice. *Proc Natl Acad Sci USA*. (1998) 95:15049–54. doi: 10.1073/pnas.95.25.15049
94. Heisler LK, Zhou L, Bajwa P, Hsu J, Tecott LH. Serotonin 5-HT2C receptors regulate anxiety-like behavior. *Genes, Brain Behav*. (2007) 6:491–6. doi: 10.1111/j.1601-183X.2007.00316.x
95. Vallorttgara G, Zanforlin M. Open-field behavior of young chicks (*Gallus gallus*): antipredatory responses, social reinstatement motivation, and gender effects. *Anim Learn Behav*. (1988) 16:359–62. doi: 10.3758/BF03209088

**Conflict of Interest:** TW holds the Egg Farmers of Canada Chair in Poultry Welfare.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 24 June 2020
doi: 10.3389/fvets.2020.00325

# Quantifying Individual Response to PRRSV Using Dynamic Indicators of Resilience Based on Activity

*Lisette E. van der Zande[1]*, Jenelle R. Dunkelberger[2], T. Bas Rodenburg[1,3], J. Elizabeth Bolhuis[1], Pramod K. Mathur[4], W. James Cairns[5], Michael C. Keyes[5], John M. Eggert[2], Erin A. Little[6], Scott A. Dee[6] and Egbert F. Knol[4]*

[1] Adaptation Physiology Group, Wageningen University & Research, Wageningen, Netherlands, [2] Topigs Norsvin USA, Burnsville, MN, United States, [3] Animals in Science and Society, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [4] Topigs Norsvin Research Center, Beuningen, Netherlands, [5] Remote Insights, Minneapolis, MN, United States, [6] Pipestone Applied Research, Pipestone, MN, United States

Pigs are faced with various perturbations throughout their lives, some of which are induced by management practices, others by natural causes. Resilience is described as the ability to recover from or cope with a perturbation. Using these data, activity patterns of an individual, as well as deviations from these patterns, can potentially be used to quantify resilience. Dynamic indicators of resilience (DIORs) may measure resilience on a different dimension by calculating variation, autocorrelation and skewness of activity from the absolute activity data. The aim of this study was to investigate the potential of using DIORs of activity, such as average, root mean square error (RMSE), autocorrelation or skewness as indicators of resilience to infection with the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). For this study, individual activity was obtained from 232 pigs equipped with ear tag accelerometers and inoculated with PRRSV between seven and 9 weeks of age. Clinical scores were assigned to each individual at 13 days post-challenge and used to distinguish between a resilient and non-resilient group. Mortality post-challenge was also recorded. Average, RMSE, autocorrelation and skewness of activity were calculated for the pre- and post-challenge phases, as well as the change in activity level pre- vs. post-challenge (i.e., delta). DIORs pre-challenge were expected to predict resilience to PRRSV in the absence of PRRSV infection, whereas DIORs post-challenge and delta were expected to reflect the effect of the PRRSV challenge. None of the pre-challenge DIORs predicted morbidity or mortality post-challenge. However, a higher RMSE in the 3 days post-challenge and larger change in level and RMSE of activity from pre- to post-challenge tended to increase the probability of clinical signs at day 13 post-infection (poor resilience). A higher skewness post-challenge (tendency) and a larger change in skewness from pre- to post-challenge increased the probability of mortality. A decrease in skewness post-challenge lowered the risk of mortality. The post-challenge DIOR autocorrelation was neither linked to morbidity nor to mortality. In conclusion, results from this study showed that post-challenge DIORs of activity can be used to quantify resilience to PRRSV challenge.

**Keywords: resilience, accelerometer, dynamic indicator of resilience, activity, pig behavior**

OPEN ACCESS

***Edited by:***
*Rebecca K. Meagher, Dalhousie University, Canada*

***Reviewed by:***
*Susan Detmer, University of Saskatchewan, Canada*
*Kaitlin Wurtz, Michigan State University, United States*

****Correspondence:***
*Lisette E. van der Zande*
*lisette.vanderzande@wur.nl*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *25 February 2020*
***Accepted:*** *11 May 2020*
***Published:*** *24 June 2020*

***Citation:***
*van der Zande LE, Dunkelberger JR, Rodenburg TB, Bolhuis JE, Mathur PK, Cairns WJ, Keyes MC, Eggert JM, Little EA, Dee SA and Knol EF (2020) Quantifying Individual Response to PRRSV Using Dynamic Indicators of Resilience Based on Activity. Front. Vet. Sci. 7:325. doi: 10.3389/fvets.2020.00325*

## INTRODUCTION

Resilience is defined as the ability to rapidly recover from or cope with a perturbation (1). Perturbations can be of any natural cause (e.g., heat stress) or can, in the case of farm animals, be induced by management practices (e.g., transportation). Pigs face multiple perturbations during their lives. When exposed to a perturbation, pigs may show individual differences in resilience. Improving resilience in pigs may contribute to sustainable pig production for a number of reasons. Resilient pigs are better able to recover from perturbations, including infectious challenges, and require fewer treatments and management interventions. The improved overall health status of resilient animals also result in improved animal welfare. In addition, because resilient pigs are less disturbed by a perturbation, they require less feed than non-resilient pigs for the same amount of growth, and therefore have a better feed efficiency (2). For these reasons, promoting resilience in pigs by optimizing (early life) conditions or by genetic selection, is desirable for future pig production.

Resilience may be measured in various ways, for instance by using physiological parameters. Blood parameters, such as white blood cell count and hemoglobin level, are examples of physiological parameters used as indicators of resilience (3). Other physiological variables used are production parameters like body weight and milk yield, which are commonly used to predict health related traits (4, 5). However, despite the number of parameters used, the lack of a golden standard for quantifying resilience remains a challenge. Assessment of physiological parameters can be invasive to the animals, and is often labor intensive. Moreover, it is often not feasible to collect physiological data repeatedly, whereas for assessment of recovery time following a perturbation, frequent, or continuous measurements are required. Behavior is one example of a non-invasive parameter with the potential for easy, repeatable observations. Weary et al. (6) stated that behavior is the most commonly used indicator for illness, as reduced activity is a main characteristic of the sickness response that is induced after infection (7), and may also occur after other stressors (8). Locomotor behavior is therefore often included in the ethogram of studies investigating illness. Traditional behavioral observation methods are labor intensive, especially when animals need to be studied frequently. Precision phenotyping tools, such as wearable accelerometers, which are capable of quantifying activity automatically, are therefore an attractive alternative. Accelerometers measure acceleration along the *x*, *y*, and *z*-axis. Using machine learning models, acceleration can be translated to activity which can, in turn, possibly be used to quantify resilience.

Apart from changes in the level of activity *per se*, dynamic changes in activity patterns may be related to resilience (9). Dynamic indicators of resilience (DIORs), which are capable of quantifying deviations in functioning of biological systems, are proposed by Scheffer et al. (10) and have been adopted for farm animals as resilience indicators (4). Such DIORs are, for instance, variance, and autocorrelation in repeatedly measured variables, which may include activity. It is expected that resilient pigs will show less variation in activity following a perturbation. In general, the activity level of pigs following a health challenge will be reduced. Pigs that recover more quickly from such a challenge (i.e., resilient pigs) will return to their initial level of activity faster than non-resilient pigs. This should result in a lower Root Mean Square Error (RMSE) of activity. Putz et al. (11) found a positive genetic correlation between RMSE of feed intake and mortality, suggesting that RMSE of feed intake can be used as an indicator of resilience. Autocorrelation represents the degree of similarity between two given time periods and ranges from −1 to 1. It is hypothesized that resilient pigs will have a (lag-1) autocorrelation of activity around zero (12), as their fast recovery results in less resemblance to previous days. Less resilient pigs recover more slowly from a perturbation, resulting in more similarity in activity of previous days for a longer period of time, i.e., a high autocorrelation. Skewness indicates the direction of the response to perturbation, i.e., a positive or negative response. It is expected that resilient pigs will have a skewness around zero as they recover more quickly from a perturbation than non-resilient pigs. All DIORs are expected to be most informative immediately following a perturbation. It can be observed directly whether a decrease in activity occurs, how steep the slope of the decrease is, and how long it persists. However, it has been suggested that dynamic patterns in repeatedly measured biological systems before a major perturbation might also be predictive of resilience. Systems losing resilience, approaching a tipping point to an alternative state (e.g., disease) may also show slower recovery from small, natural perturbations in the environment, resulting in, for instance, higher autocorrelation, and variance [see (10), for review].

In this study, DIORs based on activity were used to measure and potentially predict resilience following a Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection. PRRSV is a common infection among pig populations (13). As its name implies, PRRSV results in two main pathologies: reproductive failure and respiratory disease. Reproductive failure occurs in pregnant sows and results in abortions, mummified piglets, and weak live born piglets. Growing pigs infected with PRRSV may suffer from high fever, have loss of appetite and become lethargic or less active, leading to reduced growth and feeding efficiency, and increased mortality. The course of the clinical signs is on average 2 weeks. Despite the availability of vaccines, PRRS remains a difficult disease to control and regular outbreaks occur. Besides the impairment of pig welfare, PRRSV causes severe economic losses for the farmer.

The aim of this study was to investigate whether activity levels, or DIORs such as RMSE, autocorrelation or skewness of activity patterns, can be used as dynamic indicators of resilience following PRRSV infection in pigs.

## MATERIALS AND METHODS

Data for this paper were obtained from a subset of pigs in an experiment executed by Pipestone Veterinary Research and Topigs Norsvin USA. Prior to the start of that experiment, Pipestone Applied Research (PAR) institutional animal care and use committees (PAR IACUC 1-18) reviewed and approved the trial.

## Animals and Housing

A total of 2,186 commercial crossbred pigs from a commercial sow farm were used for the study we obtained data from. Upon weaning at approximately 3 weeks of age, pigs were shipped to a commercial research facility in the US. Each pen had fully slatted floors, with 2 cup waterers and a 4-hole dry feeder which provided 35 cm of feeder space per pig. Feed and water were provided *ad libitum*. Pigs originated from three genetic groups. Two groups were sired by boars from the same genetic line, but these boars were selected based on a different breeding goal. The third group was sired by a different genetic line. Upon arrival at the research facility, pigs were penned by genetic group and balanced by sex with 27 pigs housed per pen (0.65 $m^2$/pig) in 81 pens in total and all pigs were vaccinated per the label instructions using a PRRS modified live virus vaccine (IngelVac ATP, Boehringer Ingelheim). Pens had fully slatted concrete floors. Lights were on in the facility from 8:00 to 20:00 with a night light turned on outside of these hours. Four weeks later, pigs were experimentally inoculated with PRRS virus variant 1-7-4 at a total dose of $1 \times 10^5$ TCID50 via the IM route [SD15-174 (lineage 1)-TB3-P8, SDSU, Brookings, USA] (14). At 0, 13, and 42 days post-infection, corresponding with expected peak PRRS viremia and viral clearance at 13 and 42 days post-infection, pigs were weighted and clinical scores were assigned using a 6-point scoring system (15, 16). Scores were assigned as follows where: "1," healthy; "2," mild signs of disease; "3," moderate signs of disease; "4," advanced signs of disease; "5," extreme signs of disease; and "6," deceased (including day) (17). We could not define the recovery period using activity, because clinical scores were not assessed daily. Therefore, clinical scores at 13 days post-infection were used to distinguish pigs with a favorable or unfavorable outcome of the infection, where pigs with a clinical score of "1" were classified as "resilient," and pigs with a clinical score ">1" were classified as "non-resilient."

## Collection of Accelerometer Data

A subset of 232 pigs, originating from 9 pens (3 pens per genetic group), were equipped with individual accelerometer ear tags at 5 weeks of age (Remote Insights, Minneapolis, USA). Accelerometer data were recorded from 23 days prior to infection with PRRSV to 42 days post-infection. Videos of the pigs were annotated for activity by Remote Insights. The annotations were used as training and validation data for a machine learning model to classify their activity (Remote Insights, Minneapolis, USA). A 5-s window was classified as active or inactive, based on the output of the machine learning model, which resulted in 720 windows per hour. Data were transformed to minutes per hour. Forty-seven animals were removed from the final dataset, due to missing data for more than 20 consecutive hours, resulting in a total of 185 animals used for analyses. Missing values influence the calculation of DIORs. To avoid this, a rolling average was used for the analysis with a window of 12 h.

## DIORs Calculation

Dynamic indicators of resilience (DIORs) were calculated per individual for the pre-challenge (from 23 days pre-challenge until challenge) and post-challenge (from challenge until 3 days post-challenge) phases, as well as the change in activity level from 3 days pre-challenge vs. 3 days post-challenge (i.e., delta). Pre-challenge data were used to potentially predict resilience, based on clinical scores on day 13 post-challenge, without the influence of the PRRSV inoculation. DIORs post-challenge, based on data from the first 3 days post-challenge, were also used to potentially predict resilience and mortality. The first 3 days post-challenge were chosen, because on the fourth day post-challenge the first pig died, so all animals have data collection up to 3 days post-challenge. The delta of DIORs following inoculation was calculated by subtracting DIORs of 3 days pre-challenge from DIORs of 3 days post-challenge.

Root Mean Square Error (RMSE) of activity of the $j^{\text{th}}$ individual was calculated as:

$$RMSE_j = \sqrt{\frac{\sum_{i=1}^{n_j} (x_{f_{ij}} - x_{o_{ij}})^2}{n_j}},$$

where $x_{f_{ij}}$ is the forecasted observation $i$ of the $j^{\text{th}}$ individual, $x_{o_{ij}}$ is the observed observation $i$ of the $j^{\text{th}}$ individual, and $n_j$ is the number of observations of the $j^{th}$ individual.

Autocorrelation of activity of the $j^{\text{th}}$ individual was calculated as:

$$Autocorrelation_j = \frac{\sum_{i=1}^{n_j-k} (x_{ij} - \bar{x}_j)(x_{(i+k)j} - \bar{x}_j)}{\sum_{i=1}^{n_j} (x_{ij} - \bar{x}_j)^2},$$

Where $n_j$ is the number of observations of the $j^{\text{th}}$ individual, $x_{ij}$ the $i^{\text{th}}$ observation of the $j^{\text{th}}$ individual, and $\bar{x}_j$ the sample mean of the $j^{\text{th}}$ individual.

Skewness of activity of the $j^{\text{th}}$ individual was calculated as:

$$Skewness_j = \frac{\sqrt{n_j(n_j-1)}}{n_j - 2} \frac{m_3}{m_2^{3/2}},$$

where $n_j$ is the number of observations of the $j^{\text{th}}$ individual, $m_k = \frac{1}{n_j} \sum_{i=1}^{n_j} (x_{ij} - \bar{x}_j)^k$ , where $x_{ij}$ is the $i^{\text{th}}$ observation of the $j^{\text{th}}$ individual, and $\bar{x}_j$ the sample mean of the $j^{\text{th}}$ individual.

## Statistical Analysis

All models were fitted using R (18). A generalized linear mixed model using a binomial distribution with logit link function was used to test whether DIORs were different for resilient and non-resilient pigs (based on assigned clinical scores). DIORs were tested independent of each other. Fixed effects in the generalized linear mixed model were DIOR and clinical score at the day of inoculation as some pigs already had early or moderate signs of clinical disease. Pen was included as a random effect. Mortality was tested using Cox regression survival analysis. Fixed effects in the Cox regression model were DIOR and clinical score at the day of inoculation. Pen was included as a random effect.

# RESULTS

Two pigs had died prior to inoculation. At day 13 post-challenge, 92 pigs had a clinical score of "1" (i.e., resilient group), where

93 pigs had a clinical score of "2" or greater (i.e., non-resilient group). The resilient group had significantly ($P < 0.001$) higher average daily gain between inoculation and day 13 post-challenge compared to the non-resilient group (0.47 ± 0.02 vs. 0.23 ± 0.02 kg). At day 13 post-challenge, 7 pigs had died between 1 day pre-challenge and 12 days post-challenge. By the end of the study (at 42 days post-challenge), 13 pigs had died between 1 day pre-challenge, and 27 days post-challenge. **Table 1** shows the means and standard deviations of DIORs pre- and post-challenge, illustrating that the average activity levels decreased following challenge, whereas the impact on other DIORs was minimal.

## Association Between DIORs Pre-challenge and Morbidity and Mortality

Odds ratios given in **Tables 2**, **4** reflect the probability of being non-resilient, i.e., showing clinical signs at day 13 post infection, over the probability of being resilient. The hazard ratios presented in **Tables 3**, **5** give the probability of mortality in respect of time.

**TABLE 1 |** Means and corresponding standard deviation in parentheses for DIORs of activity (min/hour) pre-challenge and post-challenge.

| DIOR | Pre-challenge[a] | Post-challenge[b] |
|---|---|---|
| Average activity[c] | 12.17 (1.63) | 8.41 (2.00) |
| RMSE of activity[c] | 3.75 (0.60) | 3.60 (0.97) |
| Autocorrelation of activity | 0.94 (0.01) | 0.91 (0.03) |
| Skewness of activity | 0.24 (0.34) | 0.31 (0.38) |

[a] *Pre-challenge is from 23 days pre-challenge until challenge.*
[b] *Post-challenge is from challenge until 3 days post-challenge.*
[c] *In minutes per hour.*

**TABLE 2 |** Odds ratios with 95% confidence intervals (CI) for DIORs of activity pre-challenge (based on 23 days) using generalized linear mixed models for resilience (i.e. morbidity) following PRRSV inoculation.

| DIOR[a] | Odds ratio (95% CI) | *P*-value |
|---|---|---|
| Average activity | 1.14 (0.92–1.40) | 0.32 |
| RMSE of activity | 1.14 (0.66–1.97) | 0.61 |
| Skewness of activity | 0.99 (0.36–2.77) | 0.71 |

[a] *Odds ratio of autocorrelation could not be estimated. The variation in autocorrelation was minimal, resulting in very high confidence intervals.*

**TABLE 3 |** Hazard ratios with 95% confidence intervals (CI) for DIORs of activity pre-challenge (based on 23 days) using Cox regression models for mortality following PRRSV inoculation.

| DIOR[a] | Hazard ratio (95% CI) | *P*-value |
|---|---|---|
| Average activity | 1.10 (0.77–1.60) | 0.60 |
| RMSE of activity | 1.24 (0.49–3.20) | 0.65 |
| Skewness of activity | 0.27 (0.04–1.40) | 0.11 |

[a] *Hazard ratio of autocorrelation could not be estimated. The variation in autocorrelation was minimal, resulting in very high confidence intervals.*

DIORs pre-challenge did not relate to the probability of being non-resilient (**Table 2**). In addition, probability of mortality post-challenge could not be predicted by DIORs pre-challenge (**Table 3**).

## Association Between DIORs of Activity Post-challenge and Morbidity and Mortality

RMSE of activity 3 days post-challenge tended to be different between resilient and non-resilient groups (**Table 4**). The odds ratio of RMSE indicates that for every one-unit increase in RMSE, the odds of being non-resilient increases by 1.42 times. Skewness of activity tended to relate to mortality (**Table 5**). Every one-unit increase in skewness, the relative risk of mortality tended to increase 3.02 times.

## Association Between Change in DIORs From Pre- to Post-challenge and Morbidity and Mortality

The change in DIORs was calculated by subtracting the DIOR for 3 days pre-challenge from the DIOR for 3 days post-challenge. **Table 6** shows that changes in average activity and RMSE from pre-challenge to post-challenge tended to affect the probability of a non-resilient outcome of the infection. When the average activity decreased post-challenge by one-unit, the probability of being non-resilient was 22% higher (1 divided by 0.82). The effect of changes in RMSE was in the opposite direction. One-unit increase in RMSE tended to increase the odds of being non-resilient by 1.34. The change in skewness significantly affected the probability of mortality (**Table 7**). For every one-unit increase in skewness, the relative risk of mortality increased by 3.70.

**TABLE 4 |** Odds ratios with 95% confidence intervals (CI) of DIORs of activity 3 days post-challenge using generalized linear mixed model for resilience (i.e. morbidity) following PRRSV inoculation.

| DIOR[a] | Odds ratio (95% CI) | *P*-value |
|---|---|---|
| Average activity | 1.04 (0.88–1.24) | 0.65 |
| RMSE of activity | 1.42 (1.01–2.05) | 0.05 |
| Skewness of activity | 1.30 (0.56–3.04) | 0.54 |

[a] *Odds ratio of autocorrelation could not be estimated. The variation in autocorrelation was minimal, resulting in very high confidence intervals.*

**TABLE 5 |** Hazard ratios with 95% confidence intervals (CI) of DIORs of activity 3 days post-challenge using Cox regression models for mortality following PRRSV inoculation.

| DIOR[a] | Hazard ratio (95% CI) | *P*-value |
|---|---|---|
| Average activity | 0.80 (0.58–1.10) | 0.18 |
| RMSE of activity | 1.09 (0.59–2.00) | 0.78 |
| Skewness of activity | 3.02 (0.92–10.00) | 0.07 |

[a] *Hazard ratio of autocorrelation could not be estimated. The variation in autocorrelation was minimal, resulting in very high confidence intervals.*

**TABLE 6 |** Odds ratios with 95% confidence intervals (CI) of the difference in DIORs of activity pre-challenge and post-challenge using generalized linear mixed models ($n = 185$) for resilience (i.e. morbidity) groups following PRRSV inoculation.

| DIOR[a] | Odds ratio (95% CI) | *P*-value |
|---|---|---|
| Average activity | 0.82 (0.66–1.01) | 0.06 |
| RMSE of activity | 1.34 (0.98–1.87) | 0.07 |
| Skewness of activity | 1.18 (0.56–2.22) | 0.75 |

[a]*Odds ratio of autocorrelation could not be estimated. The variation in autocorrelation was minimal, resulting in very high confidence intervals.*

**TABLE 7 |** Hazard ratios with 95% confidence intervals (CI) of the difference in DIORs of activity pre-challenge and post-challenge using Cox regression models for mortality following PRRSV inoculation.

| DIOR[a] | Hazard ratio (95% CI) | *P*-value |
|---|---|---|
| Average activity | 0.79 (0.52–1.20) | 0.23 |
| RMSE of activity | 1.21 (0.66–2.20) | 0.54 |
| Skewness of activity | 3.70 (1.5–9.0) | 0.004 |

[a]*Hazard ratio of autocorrelation could not be estimated. The variation in autocorrelation was minimal, resulting in very high confidence intervals.*

## DISCUSSION

This study investigated the use of DIORs, including average, RMSE, autocorrelation, and skewness of activity to quantify resilience following PRRSV infection. It was expected that DIORs pre-challenge could be predictive of morbidity or mortality post-challenge. However, no DIOR pre-challenge was identified as predictive for morbidity or mortality in this study. Previous studies that investigated DIORs in livestock calculated DIORs using the entire study period, including the challenge period. This study identified associations between DIORs based on activity and resilience after the PRRSV challenge only, indicating that these DIORs are only associated with resilience when the animal is challenged.

To our knowledge, this is the first study to investigate pre-challenge DIORs as potential indicators of resilience in livestock. Gijzel et al. (19) explored the association between DIORs and frailty levels of elderly people. Results showed greater variation in the physical, mental, and social domain, for frail elderly individuals than non-frail elderly individuals. It should be noted, though, that in this between-subject study within-subject changes in resilience were not investigated. Thus, although DIORs pre-challenge may be associated with resilience, results from this study did not support predictive value of DIORs related to activity for the recovery of pigs from a PRRVS infection.

It was expected that activity would decrease following PRRSV inoculation, given that sickness behavior is typically characterized by a decrease in locomotor activity (20). The results from this study support this by showing that a decrease in activity post-challenge as compared with pre-challenge levels, increased the risk of being classified as non-resilient, i.e., showing clinical signs on day 13 post challenge. This suggests that changes in activity levels in the early stage of infection may be a useful DIOR following PRRSV infection. Several studies have reported a decrease in activity following PRRSV-infection (7, 21) or other diseases (22). However, occasionally, an increase in activity may be observed post-infection. For example, pigs infected with *Salmonella* were more active (23). Another perturbation, such as regrouping, is also associated with an increase in activity. After regrouping, pigs show an increase in activity (24). Therefore, the desired direction of activity changes for identifying resilient pigs may differ depending on the specific perturbation.

RMSE post-challenge and the change in RMSE following PRRSV inoculation were linked to morbidity. A higher increase in RMSE following and a higher RMSE post-challenge tended to increase the risk of a non-resilient outcome, i.e., morbidity or mortality. No associations were identified between RMSE and mortality alone, whereas Putz et al. (11) found that a higher RMSE of feed intake following natural disease challenge was associated with higher mortality. One possible explanation for this finding could be that a much lower mortality rate was observed for this study (7%) compared to the mortality rate observed by Putz et al. (11) (26%). The perturbation used by Putz et al. (11) included various viral and bacterial diseases, whereas this study used only one experimentally induced viral disease as a perturbation. Furthermore, deviations in feed intake may be more informative for mortality than deviations in activity. Another explanation could be the smaller sample size in this study.

Autocorrelation was expected to be around zero for resilient animals. However, autocorrelation had little to no variation between animals. The confidence interval of odds and hazard ratio had a range of more than one thousand (data not shown). Multiplying autocorrelation by 100 lowered the confidence interval. However, autocorrelation in activity remained uninformative regarding morbidity or mortality. Apart from the possibility that the time series resolution and length may not have been optimal for calculation of this DIOR, not all variables are characterized by critical slowing down, of which autocorrelation is a typical indicator. It has been argued that only time series of physiological variables that are maintained close to a pre-determined setpoint and fluctuate around an equilibrium, "regulated variables" exhibit critical slowing down when resilience is reduced (25). In line with this, Berghof et al. (4) and Poppe et al. (5) concluded that autocorrelation in body weight of layer chickens and milk yield of dairy cattle seem to be less informative for quantifying resilience.

In contrast with RMSE of activity, which tended to be related to morbidity, skewness in activity post-challenge, and particularly the change in skewness from pre- to post-challenge, was associated with mortality rather than morbidity. Skewness was expected to be around zero for resilient animals. Lower skewness post-challenge indeed increased the odds of being resilient. Skewness post-challenge had a mean of 0.31 (**Table 1**), so a decrease in skewness indicates a movement toward zero. However, skewness has a range of −1 to 1, so a one-unit shift in skewness is very unlikely. Berghof et al. (4) and Poppe et al. (5) concluded that skewness in body weight of layer chickens and milk yield was less informative for health and longevity traits than other DIORs. This is also in line with the findings from this

study, which indicate that skewness is not related to morbidity. Skewness could be sensitive to outliers, which could be the case for individual recordings of milk yield and activity (5). Results from this study did, however, identify an association between reduced skewness (movement toward zero) with decreased risk of mortality.

For young animals, activity decreases over time irrespective of a perturbation (26). This study did not correct for this decrease in activity. DIORs post-challenge and their deviations from pre-challenge values were calculated based on 3 days, and it is therefore assumed that the changes in these 3 days are due to the perturbation. To use activity of the whole period, control animals should be added to be able to correct for the decrease in activity due to aging.

The results obtained from this study demonstrated the value of DIORs based on activity to quantify resilience to disease challenge in pigs, although studies with larger sample sizes are needed to confirm this. The accelerometers used in this study measured acceleration using three axes and machine learning models to calculate activity, which is a black box approach. Based on accelerations, activity could be assessed, but spatial distribution, specific behaviors (e.g., whether a pig was shaking its head or running around) or social interactions could not be measured. Conversely, computer vision, allowing for immediate identification of a pig in a video and registering of its coordinates, could be used to extract the location and specific behavior of the animal. Additional information captured using computer vision might include distance moved, velocity, spatial distribution, and social interactions. Taken together, these parameters would allow for the analysis of more complex activity and behavioral traits. Therefore, data generated via computer vision technology may improve estimation of DIORs, compared to using accelerometer data. However, accelerometers are currently commercially available, while camera technology is not yet ready for implementation at the commercial level. In the future, the cost/benefit of accelerometers vs. cameras will need to be evaluated on a case-by-case basis.

## CONCLUSION

Results from this study showed that DIORs based on activity pre-challenge could not predict morbidity and mortality following a PRRSV infection. However, RMSE in the 3 days post-challenge and the change in RMSE and average activity from pre-to post-challenge tended to be associated with morbidity 13 days after infection. Skewness post-challenge tended to be associated with mortality, and the change in skewness was significantly related to mortality. Thus, DIORs based on activity showed their value to quantify resilience to a disease challenge. To explore the full potential of DIORs more in depth, more elaborate measurements of behavior are desirable. Computer vision may allow for these in-depth measurements which cannot be assessed using accelerometers.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

This animal study was reviewed and approved by Pipestone Applied Research (PAR) institutional animal care and use committees (PAR IACUC 1-18).

## AUTHOR CONTRIBUTIONS

JD, PM, JE, EL, SD, and EK designed the experiment and developed protocols for animal sourcing, management, and phenotype recording. WC and MK employed the ear tag accelerometers and generated the activity dataset. LZ analyzed the data and wrote the manuscript with help of TR, JB, JD, and EK. All authors reviewed and approved the final manuscript.

## FUNDING

This study was part of research program Green II—Towards a Groundbreaking and future-oriented system change in agriculture and horticulture with Project No. ALWGR.2017.007 and was financed by the Netherlands Organization for Scientific Research.

## ACKNOWLEDGMENTS

We thank Ingrid van de Leemput and Egbert van Nes of Wageningen University & Research Aquatic Ecology and Water Quality Management for their inspiration to use a rolling window to deal with missing values.

## REFERENCES

1. Colditz IG, Hine BC. Resilience in farm animals: biology, management, breeding and implications for animal welfare. *Anim Product Sci.* (2016) 56:1961–83. doi: 10.1071/AN15297
2. Hermesch S, Li L, Doeschl-Wilson A, Gilbert H. Selection for productivity and robustness traits in pigs. *Anim Product Sci.* (2015) 55:1437–47. doi: 10.1071/AN15275
3. Hermesch S, Luxford B. Genetic parameters for white blood cells, haemoglobin and growth in weaner pigs for genetic improvement of disease resilience. In: *Proceedings of the 11th World Congress on Genetics Applied to Livestock Production*. Auckland (2018). p. 11–16.
4. Berghof T, Bovenhuis H, Mulder H. Body weight deviations as indicator for resilience in layer chickens. *Front Genet.* (2019) 10:1216. doi: 10.3389/fgene.2019.01216
5. Poppe M, Veerkamp R, Van Pelt M, Mulder H. Exploration of variance, autocorrelation, and skewness of deviations from lactation curves as resilience indicators for breeding. *J Dairy Sci.* (2020) 103:1667–84. doi: 10.3168/jds.2019-17290
6. Weary D, Huzzey J, Von Keyserlingk M. Board-invited review: using behavior to predict and identify ill health in animals. *J Anim Sci.* (2009) 87:770–7. doi: 10.2527/jas.2008-1297
7. van Dixhoorn ID, Reimert I, Middelkoop J, Bolhuis JE, Wisselink HJ, Koerkamp PWG, et al. Enriched housing reduces disease susceptibility to

co-infection with porcine reproductive and respiratory virus (PRRSV) and Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*) in young pigs. *PLoS ONE*. (2016) 11:e0161832. doi: 10.1371/journal.pone.0161832
8. Costa A, Ismayilova G, Borgonovo F, Viazzi S, Berckmans D, Guarino M. Image-processing technique to measure pig activity in response to climatic variation in a pig barn. *Anim Product Sci.* (2014) 54:1075–83. doi: 10.1071/AN13031
9. van Dixhoorn I, de Mol R, van der Werf J, van Mourik S, van Reenen C. Indicators of resilience during the transition period in dairy cows: a case study. *J Dairy Sci.* (2018) 101:10271–82. doi: 10.3168/jds.2018-14779
10. Scheffer M, Bolhuis JE, Borsboom D, Buchman TG, Gijzel SM, Goulson D, et al. Quantifying resilience of humans and other animals. *Proc Natl Acad Sci USA*. (2018) 115:11883–90. doi: 10.1073/pnas.1810630115
11. Putz AM, Harding JC, Dyck MK, Fortin F, Plastow GS, Dekkers JC, et al. Novel resilience phenotypes using feed intake data from a natural disease challenge model in wean-to-finish pigs. *Front Genet.* (2018) 9:660. doi: 10.3389/fgene.2018.00660
12. Berghof T, Poppe M, Mulder H. Opportunities to improve resilience in animal breeding programs. *Front Genet.* (2019) 9:692. doi: 10.3389/fgene.2018.00692
13. Almeida M, Zimmerman JJ, Wang C, Linhares DC. Assessment of abattoir based monitoring of PRRSV using oral fluids. *Prev Vet Med.* (2018) 158:137–45. doi: 10.1016/j.prevetmed.2018.08.002
14. Dee S, Guzman JE, Hanson D, Garbes N, Morrison R, Amodie D, et al. A randomized controlled trial to evaluate performance of pigs raised in antibiotic-free or conventional production systems following challenge with porcine reproductive and respiratory syndrome virus. *PLoS ONE*. (2018) 13:e0208430. doi: 10.1371/journal.pone.0208430
15. Lopez O, Osorio F. Role of neutralizing antibodies in PRRSV protective immunity. *Vet Immunol Immunopathol.* (2004) 102:155–63. doi: 10.1016/j.vetimm.2004.09.005
16. Hess AS, Islam Z, Hess MK, Rowland RR, Lunney JK, Doeschl-Wilson A, et al. Comparison of host genetic factors influencing pig response to infection with two North American isolates of porcine reproductive and respiratory syndrome virus. *Genet Select Evol.* (2016) 48:43. doi: 10.1186/s12711-016-0222-0
17. Pantoja LG, Kuhn M, Hoover T, Amodie D, Weigel D, Dice C, et al. Impact of a Husbandry Education Program on nursery pig mortality, productivity, and treatment cost. *J Swine Health Product.* (2013) 21:188–94.
18. R Core Team. *R: A Language and Environment for Statistical Computing* (2013).
19. Gijzel SM, van de Leemput IA, Scheffer M, Roppolo M, Olde Rikkert MG, Melis RJ. Dynamical resilience indicators in time series of self-rated health correspond to frailty levels in older adults. *J Gerontol Ser A*. (2017) 72:991–6. doi: 10.1093/gerona/glx065
20. Hart BL. Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev.* (1988) 12:123–37. doi: 10.1016/S0149-7634(88)80004-6
21. Escobar J, Van Alstine WG, Baker DH, Johnson RW. Behaviour of pigs with viral and bacterial pneumonia. *Appl Anim Behav Sci.* (2007) 105:42–50. doi: 10.1016/j.applanim.2006.06.005
22. Reiner G, Hübner K, Hepp S. Suffering in diseased pigs as expressed by behavioural, clinical and clinical–chemical traits, in a well defined parasite model. *Appl Anim Behav Sci.* (2009) 118:222–31. doi: 10.1016/j.applanim.2009.02.010
23. Rostagno MH, Eicher SD, Lay Jr DC. Immunological, physiological, and behavioral effects of *Salmonella enterica* carriage and shedding in experimentally infected finishing pigs. *Foodborne Pathog Dis.* (2011) 8:623–30. doi: 10.1089/fpd.2010.0735
24. Camerlink I, Turner SP, Bijma P, Bolhuis JE. Indirect genetic effects and housing conditions in relation to aggressive behaviour in pigs. *PLoS ONE*. (2013) 8:e65136. doi: 10.1371/journal.pone.0065136
25. Gijzel S. *Bouncing back: Using a complex dynamical systems approach to measure physical resilience in older adults* (dissertation). Radboudumc, Nijmegen, Netherlands (2019).
26. Bolhuis JE, Schouten WG, Schrama JW, Wiegant VM. Behavioural development of pigs with different coping characteristics in barren and substrate-enriched housing conditions. *Appl Anim Behav Sci.* (2005) 93:213–28. doi: 10.1016/j.applanim.2005.01.006

**Conflict of Interest:** JD, PM, JE, and EK were employed by Topigs Norsvin. WC and MK were employed by Remote Insights. EL and SD were employed by Pipestone Applied Research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

METHODS
published: 13 August 2020
doi: 10.3389/fvets.2020.00523

# Mind the Queue: A Case Study in Visualizing Heterogeneous Behavioral Patterns in Livestock Sensor Data Using Unsupervised Machine Learning Techniques

*Catherine McVey*[1], *Fushing Hsieh*[2], *Diego Manriquez*[3], *Pablo Pinedo*[3] *and Kristina Horback*[1]*

[1] Department of Animal Science, University of California, Davis, Davis, CA, United States, [2] Department of Statistics, University of California, Davis, Davis, CA, United States, [3] Department of Animal Science, Colorado State University, Fort Collins, CO, United States

OPEN ACCESS

**Edited by:**
Rebecca K. Meagher, Faculty of Agriculture, Dalhousie University, Canada

**Reviewed by:**
Young Chang, Faculty of Agriculture, Dalhousie University, Canada
Helen R. Fielding, University of Edinburgh, United Kingdom

***Correspondence:**
Kristina Horback
kmhorback@ucdavis.edu

**Specialty section:**
This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science

**Received:** 03 March 2020
**Accepted:** 07 July 2020
**Published:** 13 August 2020

**Citation:**
McVey C, Hsieh F, Manriquez D, Pinedo P and Horback K (2020) Mind the Queue: A Case Study in Visualizing Heterogeneous Behavioral Patterns in Livestock Sensor Data Using Unsupervised Machine Learning Techniques. Front. Vet. Sci. 7:523. doi: 10.3389/fvets.2020.00523

Sensor technologies allow ethologists to continuously monitor the behaviors of large numbers of animals over extended periods of time. This creates new opportunities to study livestock behavior in commercial settings, but also new methodological challenges. Densely sampled behavioral data from large heterogeneous groups can contain a range of complex patterns and stochastic structures that may be difficult to visualize using conventional exploratory data analysis techniques. The goal of this research was to assess the efficacy of unsupervised machine learning tools in recovering complex behavioral patterns from such datasets to better inform subsequent statistical modeling. This methodological case study was carried out using records on milking order, or the sequence in which cows arrange themselves as they enter the milking parlor. Data was collected over a 6-month period from a closed group of 200 mixed-parity Holstein cattle on an organic dairy. Cows at the front and rear of the queue proved more consistent in their entry position than animals at the center of the queue, a systematic pattern of heterogeneity more clearly visualized using entropy estimates, a scale and distribution-free alternative to variance robust to outliers. Dimension reduction techniques were then used to visualize relationships between cows. No evidence of social cohesion was recovered, but Diffusion Map embeddings proved more adept than PCA at revealing the underlying linear geometry of this data. Median parlor entry positions from the pre- and post-pasture subperiods were highly correlated ($R = 0.91$), suggesting a surprising degree of temporal stationarity. Data Mechanics visualizations, however, revealed heterogeneous non-stationary among subgroups of animals in the center of the group and herd-level temporal outliers. A repeated measures model recovered inconsistent evidence of a relationships between entry position and cow attributes. Mutual conditional entropy tests, a permutation-based approach to assessing bivariate correlations robust to non-independence, confirmed a significant but non-linear

association with peak milk yield, but revealed the age effect to be potentially confounded by health status. Finally, queueing records were related back to behaviors recorded via ear tag accelerometers using linear models and mutual conditional entropy tests. Both approaches recovered consistent evidence of differences in home pen behaviors across subsections of the queue.

**Keywords: milking order, exploratory data analysis, unsupervised machine learning, data mechanics, entropy, manifold learning, precision livestock**

## INTRODUCTION

For much of its history, ethological research in livestock has relied on human observers to encode behaviors of interest (1). While developing a detailed ethogram and observer training protocols constitute no simple task, there are several inherent advantages to this approach for subsequent statistical analyses. Continuous involvement of a human in the incoming data stream allows many erroneous data points to be identified and excluded from downstream analyses that they might otherwise destabilize. Extensive involvement of research personnel in the data collection phase also nurtures a deeper familiarity with the system under study. This not only aids in the specification of an appropriate statistical model and interpretation of results, but is often critical in identifying unexpected behavioral patterns that can inspire novel hypotheses.

Unfortunately, the inherent quality of such data imposes practical limitations on the quantity that can be produced. This can restrict both the number of animals utilized in a study and the period of time over which they are observed. The later limitation can overlook important dynamic features of the behavioral patterns under consideration. Restrictions on the number of animals that can be studied, on the other hand, can fundamentally alter the behavioral mechanisms at play in a herd. For example, the linearity of dominance hierarchies are known to change with group size (2). As commercial herds and flocks become ever larger, this only serves to broaden the gap between experimental findings and the welfare challenges they are meant to inform. Subsampling of animals or observations windows may be employed to reduce the number of observations collected without restricting the size of the study system. If the pre-existing base of scientific literature does not provide clear guidance on the selection of target animals or focal periods, however, such strategies may risk overlooking finer-grain behavioral patterns and skewing inferences about the collective behavior of the group (3, 4).

In recent years, livestock sensor technologies have become a popular alternative to visual observation (5–8). While the behaviors recorded are neither as complex or as detailed as those quantified via an observational ethogram, such devices have the capacity to continuously monitor hundreds or even thousands of animals for extended periods of time. Such a substantial expansion in the bandwidth capacity of ethological studies creates many new opportunities to better understand the behavior of livestock, particularly in large-scale commercial settings, but also raises new methodological challenges. Replacing nuanced human intuition with basic computer logic may increase the risk of erroneous data points, an issue that is only further compounded by the scale of data produced by such technologies, which renders many conventional visualizations techniques ineffective in identifying outliers. Observations recorded over extended time periods with high sampling frequency from large heterogeneous social groups may also contain a range of complex stochastic features—autocorrelation, temporal non-stationary, heterogeneous variance structures, non-independence between experimental units, etc. —that can lead to spurious inferences when not appropriately specified in a conventional liner model. The hands-off and somewhat black-boxed nature of many sensor platforms, however, do not nurture the intuition needed to identify many of these model structures *a priori*. Such insights must instead be drawn directly from the data itself, but here again, standard visualization tools may not scale to such large datasets.

Unsupervised machine learning (UML) tools offer a distinct empirical approach to knowledge discovery that are purpose built for large and complex datasets (9). Whereas, conventional linear models excel at providing answers to targeted experimental hypotheses, UML algorithms strive to identify and characterize the non-random patterns hiding beneath the stochastic surface of a dataset using model-free iterative techniques that impose few structural assumptions. This open-ended and highly flexible approach to data exploration may offer an empirical means by which to recover much of the familiarity with a study system that is lost with the shift from direct observation to sensor platforms. The purpose of this research was contrast the behavioral insights gleaned from UML algorithms with those recovered using conventional exploratory data analysis (EDA) techniques, and to then explore how such information could be best integrated into standard linear analysis pipelines.

Milking order, or the sequence in which cows enter the parlor to be milked, is recorded in all RFID (Radio Frequency Identification) equipped milking systems, making such records one of the most universal automated data streams to be found on modern dairies. Despite their ubiquity, such records are seldom used to inform individual or herd-level management strategies. This lack of utility, however, has not been for lack of study. Milking order has been the subject of scientific study since 1950's (10), with early investigators speculating that such records might contain pertinent information about individual cow productivity (11, 12), health (13), and social status (12, 14, 15). The modest base of scientific literature that has since been compiled on this topic, however, has struggled to recover repeatable evidence

of such associations (16–19). While such inconsistency may simply reflect non-uniformity in the behavioral strategies driving queueing patterns across different herds and farm environments, misspecification of the linear models used to describe this system could also contribute to volatility in these statistical inferences. The objective of this methodological case study will be visualize the various stochastic aspects of such records using UML tools in an effort to identify erroneous data points and heterogeneous variance structures that may not be recovered using conventional EDA techniques.

# METHODS

## Study Animal Management

Data for this case study was repurposed from a feed trial assessing the effect of an organic fat supplement on cow health and productivity through the first 150 days of lactation (20). All animal handling and experimental protocols were approved by the Colorado State University Institution of Animal Care and Use Committee (Protocol ID: 16-6704AA). The study ran from January to July of 2017 on a certified organic dairy in Northern Colorado. A total of 200 mixed-parity Holstein cows were enrolled over a 1.5 month period as study-eligible animals calved. Cows were maintained in a closed herd for the duration of the study, with sick animals temporarily removed to a hospital pen when necessary. The study pen was an open-sided free stall barn, stocked at just above half capacity with respect to bunk space and beds, with free access to an adjacent outdoor dry lot. At roughly the midpoint of the trial, cows were moved overnight to a grass pasture that conformed with organic grazing requirements [for more details on pen setup see (21)]. Cows had access to total mixed ration (TMR) ration following each milking. Animals were temporarily split into two subsections of the pen following the morning milking to facilitate administration of control and treatment diets. Cows remained locked for roughly 45 min following this division so that farm and research staff could collect health and fertility data. Additionally, all animals were fitted with CowManager® ear tag accelerometers (Agis Automatisering BV, Harmelen, Netherlands). This commercial sensor platform, while designed and optimized for disease and heat detection, also provided hourly time budget estimates for total time (min) engaged in a range of behaviors—eating, rumination, non-activity, activity, and high activity—as well as average skin temperature.

## Data Wrangling

Raw milk logs were exported from the rotary parlor following each morning milking (ALPRO, DeLaval, Tumba, Sweden), and were processed using data wrangling tools available in R version 3.5.1 (22). To account for missing records due to illnesses and RFID reader errors, ordinal entry positions were normalized by the total number of cows recorded in a given milking (18). Transforming the data to an entry quantile served to make the domain restriction uniform across days. Additionally, "dividing out" daily variations in herd size served to prevent this uncontrolled experimental factor from artificially increasing individual variability in entry position. For example, if a cow were always the last animal to enter the parlor, her ordinal entry position might vary widely with herd size, but her entry quantile would always be 1.

The first 55 days of records were excluded from analyses to allow all animals to enter the herd over the rolling enrollment period and become established in their parlor entry position (16). To avoid irregularities in cow movements, several observation days surrounding management changes were also dropped, including: the 2 days preceding transition to pasture, the 4 days following pasture access, and the final 7 days on trial. Any days where <75% of the herd was successfully recorded in the parlor were also dropped. This left a total of 80 days of milk order observations—26 recorded while cows remained overnight in their pen, and 54 after the transition to overnight pasture. Finally, cows that were not present in at least 50% of the remaining milkings were excluded from further analysis. Of the 177 cows with sufficient records, 114 had no recorded health events.

## Quantifying Degree of Randomness

The first step in understanding this system was to determine if there was any evidence of non-random patterns in queue formation. Had this data been collected observationally, researchers might have simply noted if the same cows consistently entered the parlor in a given section of the queue. Standard summary statistics do not readily lend themselves to making an equivalent empirical determination, however, as cow identity is a discrete variable with no inherent nominal value. This issue is encountered fairly regularly in ethological studies, where many qualitative behaviors have no natural ordering, such as: locations occupied in a pen at discrete time points, conspecifics an individual interacts with, feedstuffs or enrichment items engaged with, etc. Here we use entropy to quantify the stochasticity of cow-membership within each fixed quantile range ($H_q$). To compute these values, the queue was divided into 20 equally-sized segments ($q_{0-0.05}, ..., q_{0.95-1}$). For each queue segment, counts were generated to determine how frequently each individual cow had been observed in that range of entry quantiles. Shannon's entropy was then calculated conditional of queue segment (q) using the formula in Equation 1 (23).

$$H_q = -\sum_{c\,=\,cow} \frac{n_{c|q}}{N_q} * \log_2\left(\frac{n_{c|q}}{N_q}\right) \tag{1}$$

$$n_{c|q} = \textit{total times a given cow } (c) \textit{ is observed in quantile position } (q)$$

$$N_q = \textit{total animals observed in queue segment } (q) \textit{ across observed milkings}$$

With this metric, the more consistently a smaller set of cows are observed in a given segment of the queue, the smaller the entropy values becomes to reflect less stochasticity in the system. In standard statistical models, the nominal value of estimators such as loglikelihood and AIC scale with the size of the data set, and must be interpreted relative the value of equivalent

terms assessed against a null model. Analogously, the nominal value of the entropy estimates scales with the number of discrete categories used. The maximum theoretical value occurs when no underlying deterministic structures are present and all categories are equally likely to occur, which algebraically simplifies to the log of the number of discrete categories used (23). Here the maximum theoretical entropy value would be $\log_2(114) = 6.83$. To visually contrast differences in stochasticity across the queue, the observed entropy values were plotted against the median entry quantile of the corresponding queue segment using the *ggplot2* package, with maximum theoretical entropy added as a horizontal reference line (24).

Non-random patterns in queue formation could also be explored by tracking the entry position of individual cows over time. As entry quantile has a numerical value, we can now also use variance to quantify and contrast stochasticity between animals. As with all analytical approaches reviewed in this paper, there are both strengths and shortcomings to either approach (**Table 1**). In this system there are two potential drawbacks to this conventional summary statistic. The first is that variance estimates are quite sensitive to outliers, making it difficult to empirically distinguish between cows that occupy a wider range of queue positions and animals who typically occupy a narrower range but might have gotten jostled far from their normal position on one or several occasions. The second drawback is that, because variance quantifies dispersion about a central value, it cannot distinguish between cows that demonstrate little consistency in entry position and multimodal queuing patterns. For example, if a cow always entered the parlor either first or last, we would intuitively determine that this pattern is non-random, but the corresponding variance estimate would be the largest in the herd. These issues are circumvented, however, by discretizing entry quantile values and again using entropy to quantify stochasticity. To evaluate an individual cow's variability in quantile range-memberships ($H_c$), count data was used to recalculate Shannon's entropy conditional on cow ID (c) using Equation 2 (23).

$$H_c = -\sum_{q\,=\,Queue\ Segment} \frac{n_{q|c}}{N_c} * \log_2\left(\frac{n_{q|c}}{N_c}\right) \qquad (2)$$

$n_{q|c}$ = *total times a given cow* ($c$) *is observed in quantile position* ($q$)
$N_c$ = *total number of days a given cow* ($c$) *was observed in the queue*

Here the maximum possible entropy value, signifying a cow is equally likely to occupy any queue segment, would be $\log_2(20) = 4.32$. Observed entropy and variance values were visually compared using the *ggplot2* package (24). To test if an individual cow demonstrated less stochasticity in entry positions than would be expected with a purely random queueing process, entry quantile values were again permuted within each observation day, and both variance and entropy recalculated. This process was repeated over 5,000 iterations to generate empirical cumulative density functions (CDFs) for both stochasticity estimators under the null, which were then used to estimate *p*-values for the corresponding observed values.

## Visualization of Inter-animal Relationships

Having recovered evidence of non-random patterns, the next step was to begin characterizing the behavioral mechanisms driving this heterogeneity. The most fundamental question that need be answered to inform further analysis was the degree to which queueing patterns were driven by individual or collective behaviors. Because cows jockey for position with one another in the crowd pen, where they are pushed up to enter the parlor, we know intuitively that entry quantile records cannot be considered truly independent observations. If cows move through this melee as independent agents, such that their position within the queue is determined by individual attributes—preferences, dominance, etc. —then a linear model may still provide a reasonable approximation of the underlying system. Early observational work on milking order, however, has suggested that cows may form consistent associations when entering the milking parlor, particularly when heifers are reared together (13, 25). If cows move into the parlor in cohesive units, such that queue position is more determined by clique-level than individual attributes, then network analyses may be a more appropriate.

Principal Component Analysis (PCA) is commonly employed to visualize relationships between observational units in high dimensional datasets. In this approach, redundancy between variables, here each milking record, is captured using either covariance or correlation assessed across all data points, here all animals. An eigenvector decomposition is then used to linearly compress the information contained in the data via rotation of the orthogonal axes. New axes (loadings) are added iteratively such that each new dimension is pointed in the direction of greatest remaining variability until only noise remains (26). Each data point is then projected into the resulting low-dimension linear space (27). PCA was here performed only on animals with no recorded health in order to prevent any anomalous queuing behaviors recorded from acutely or chronically ill animals from obscuring the queuing patterns of the broader herd. The correlation matrix was constructed using all pairwise complete observations, and a scree plot was used to determine the dimensionality of the resulting space (see **Supplemental Materials**). The *plotly* package (28) was then used to visualize the final embedding.

While PCA provides a computationally expedient means of visualizing high dimensional data, the underlying assumption of linearity is not always appropriate (26, 27). In some data sets complex geometric constraints, such as those commonly found with images or raw accelerometer data, and other latent deterministic features may project data points onto high dimensional geometric surfaces collectively called manifolds (29, 30). When these topologies are non-linear (cones, spheres, donuts, etc.), the spatial relationships between data points cannot always be reliably maintained when projected directly into a linear (Euclidean) space, which can lead to incorrect inferences (27, 31). Imagine, for example, you had a round globe of the world and wanted instead a flat map. Applying PCA to this task would be analogous to smooshing the globe flat on

**TABLE 1 |** Summary of analytical approaches compared in this manuscript, and a comparison of their relative strengths and shortcomings.

| Analytical Goal | Approach | Strengths | Shortcomings |
|---|---|---|---|
| Quantifying randomness | Variance | • Uses continuous measures | • Cannot use categorical data<br>• Assumes unimodality<br>• Sensitive to outliers |
| | Entropy | • Uses categorical data<br>• Permits Multimodality<br>• Robust to Outliers | • Continuous measures must be discretized, which can result in loss of information |
| Visualizing inter-animal relationships | PCA | • No metaparameters<br>• Assess embedding via loadings | • Assumes latent structures are linear (additive) |
| | Diffusion map | • No linearity assumption | • Employs metaparameters<br>• Embedding qualitatively assessed using visualizations |
| Visualizing temporal non-stationarity | Residual plots of stational repeated measures model | • Considers all data points simultaneously | • Non-homogeneous temporal trends may be overlooked |
| | Time × Response scatter plots for each cow | • Easy to create and visually assess for non-stationarity | • Difficult to contextualize trends across entire herd |
| | Data mechanics plot | • Simultaneously visualize social and temporal structure<br>• Non-homogeneous temporal trends visually enhanced | • Row and column cluster granularity must be determined visually |
| Association between queue position and cow attributes | Linear mixed effect model | • Targeted hypotheses<br>• Simultaneous estimation of multiple covariates | • Non-independence between animals inflates rate of Type I Errors |
| | Mutual conditional entropy permutation test | • Robust to between-animal non-independence<br>• Detects non-linear patterns | • Cannot adjust for influence of other variables (confounders) |
| Association between queue position and accelerometer logs of home pen behavior | Linear mixed effect model | • Targeted hypotheses | • Convergence issues with two large sets of repeated measures |
| | Mutual conditional entropy permutation test | • Generalized pattern detection<br>• Easily extended to large data | • Cannot adjust for confounding variables |

a table. Some of the original geographic relationships would be discernable, but some locations would appear erroneously close, and some landscapes would be entirely obscured. Modern manifold learning algorithms strive to more reliably project the complex geometric relationships between observational units into a standard Euclidean space by approximating the surface of a non-linear manifold with a series of interconnected flat surfaces that can then be "unwrapped" onto a linear space (32). Returning to the previous pedagogical metaphor, this would be analogous to taking pictures of the globe centered around a number of key geographic locations, and then attempting to arrange the overlapping images onto a flat table. Some geographic features will still be lost, particularly over sparsely sampled regions like the oceans, but the spatial relationships between landmarks would collectively prove more representative of the original topography.

To further explore the underlying structure of this data absent assumptions of linearity, and thereby potentially accommodate any complex geometric constraints imposed on milk order records by latent social structures within the herd, a diffusion map algorithm was implemented using functions provided in base R (22). This was done here by first calculating the Euclidean distance between temporally aligned vectors of parlor entry quantiles for each pairwise combination of cows, scaled to adjust for missing records, and then inverting these values to create a similarity matrix. From this similarity matrix a weighted network was created by progressively adding links for the $k = 10$ nearest neighbors surrounding each data point. A spectral value decomposition was then performed on the corresponding graph Laplacian matrix (27, 33). The resulting eigenvalues were used to select the appropriate number of dimensions, and the corresponding eigenvectors visualized using the 3D scatter tools from the *plotly* package (28). Finally, as a means of comparing geometric structures identified in the observed dataset with those of a completely randomized queuing process, the permutated dataset generated in the previous section was also embedded and visualized using *plotly* graphics (28).

## Characterization of Temporal Dynamics

Having determined from the previous visualizations that a linear model might be a reasonable representation of the underlying deterministic structures of this system, the next step was to explore the temporal dynamics of this dataset. In a standard repeated measures model, multiple observations from the same animal are assumed to be identically and independently sampled, implying that sampling order should not affect the observed value. If the observation period is sufficiently long to allow the underlying process to shift or evolve over time, however, stationarity cannot be assumed. Failure to statistically

accommodate a temporal trend can not only lead to spurious inferences due to incorrect estimation of error variance, but also risks overlooking dynamic features of the behaviors under consideration (34). In practice temporal trends are often assessed by first fitting a stationary model and analyzing the resulting residuals. This may suffice when the temporal trend is uniform across animals, but risks overlooking more complex non-homogeneous temporal affects. This could occur if only a subset of the larger group displays a non-stationary pattern, a risk that is likely heightened in large socially heterogeneous groups. In this physically constrained system, where we know that every cow moving forwards in the queue must force other cows backwards, compensatory trends could also be easily overlooked in collective assessment of residuals.

We first assessed temporal trend using two conventional EDA techniques. First, the *ggplot2* package (24) was used to generate scatter plots of entry quantile values against the corresponding observation date for each individual cow, with pasture access annotated with a vertical line. Plots were visually inspected for non-stationary, and are provided in **Supplemental Materials**. Next, to further explore the impact of the shift from pen to overnight pasture access on morning queueing patterns, median queue positions from the two subperiods were plotted against using the *ggplot2* package (24), and Pearson correlation (R) and Kendall Tau ($\tau$) were computed using the *stats* package (22). While these preliminary visualizations were easy to both generate and interpret, both treat cows as independent and somewhat isolated units. With such a large number of animals to consider, the capacity for human pattern detection is quickly overwhelmed, making it difficult to contextualize trends within the broader herd. Further, this approach fails to leverage non-independence between animals entering the parlor, and thus risks overlooking subtler collective responses.

Data mechanics visualizations were implemented to simultaneously explore systematic heterogeneity in milk entry quantiles both between animals and across the temporal axis. This was done by first using entry quantile values to compute two Euclidean distance matrices: one quantifying the similarity between pairwise combinations of cows, the second quantifying similarity between pairwise combinations of daily milking sequences. These distance matrices were then used to generate two independent hierarchical clustering trees using the Ward D2 method (22, 26). By cutting both trees at a fixed number of clusters, observation days and cows were both partitioned into empirically defined categories, and a contingency table was then formed with cow clusters as the row variable and day clusters as the column variable. The original distance matrices were then updated, using the clustering structure between cows to create a weighted distance matrix between days and vice versa, thereby allowing mutual information to be shared between the temporal and social axes of the dataset (see **Supplemental Materials** for details). After several iterations of this algorithm, clusterings converged toward a contingency table with minimal entropy, wherein the entry quantile values within each cell were as homogenous as possible. When the entry quantile values were subsequently visualized using a heat map, this highly generalizable entropy minimization technique served to visually enhance heterogeneity within the data driven by non-random patterns along either axis. Further, by facilitating the transfer of information between axes, interaction effects between the social and temporal dimensions of this system were magnified, which here provided a means to explore non-homogeneous temporal non-stationary between subgroups within the herd (35–37).

The data mechanics pipeline was used to analyze the temporal dynamics present in both the complete milking order dataset and the subset of animals with no recorded health events. Heat map visualizations were generated using the *pheatmap* package in R (38), with observation days arranged on the column axis and Cow ID's arranged on the row axis. Fixed values for the number of clusters used to divide the row and column axes could not be determined *a priori*. Instead this algorithm was applied on a grid from 1 to 10 clusters for either axis. The resulting 100 heat maps scanned visually to determine the clustering granularity required to bring into resolution any interactions between social and temporal mechanisms. While this process may be computationally cumbersome, it is empirically analogous to systematically varying the focus of a light microscope to bring into resolution microbes of unknown size—a tedious but effective means of identifying all relevant structures within a sample (35). Finally, the *RColorBrewer* package (39) was used to add color annotations to the column margin, to clarify temporal patterns, and to the row margins, which served to visualize potential relationships between queue position, a selection of individual cow attribute variables, and the onset of recorded health complications.

## Linear Analysis of Cow Attributes

Having thoroughly characterized the stochastic structures present in this dataset, the insights gleaned from the preceding visualizations were incorporated into a linear model to evaluate the relationship between queue position and several cow attributes. The 4 days identified as outliers by the data mechanics visualizations were first removed and the dataset converted to long format to be analyzed as a repeated measures model using the *nlme* package (40). Cow was fit as a random intercept via maximum likelihood method. Guided by the results of entropy and data mechanics visualizations, VarIdent was used to estimate separate error variance terms for each cow, and the necessity of this data-hungry heterogeneous variance model confirmed via likelihood ratio test against the null model with homogenous variance (34). After centering and scaling cow attribute variables, linear fixed effects were added for cow age (days old at start of trial), calving date (approximately the date of entry into the herd), and peak milk yield (estimated via the 95th quantile of each cow's 150 day parlor lactation record). Interaction effects were created for each combination of these linear terms, and a categorical effect added for the control and treatment groups of the fat supplementation trial. Models were generated for both the complete dataset and the subset of animals with no recorded health events, which consisted of 160 and 104 cows, respectively after removing animals with incomplete attribute records. The predictive value of each fixed effect term was evaluated via a Wald's test. Where a significant association was identified at the standard $\alpha = 0.05$ (Type I Error) confidence level, this pattern

was visualized by plotting the cow attribute variable against the predicted queue position for each cow (fixed effect + BLUP).

While UML insights served to improve the specification of model variance structures within-animal, the validity of statistical insights made at the between-animal level is still contingent upon the correct estimation of model degrees of freedom. A fundamental assumption of frequentist tests is that observations must be independently sampled. When observations are not independent, the effective degrees of freedom present in the model may be lower than the nominal value. This causes the model to be overconfident in its estimation of error terms, increasing the risk of a false positive result. Non-independence due to repeated sampling (pseudoreplication) has here been accounted for by fitting a random effect for each cow, but non-independence between animals has not been accommodated. The results of the diffusion map and data mechanics visualizations did not recover overwhelming evidence of coordinated movements between animals through the queue, which would have signified non-independence due to social cohesion (positive interclass correlation between animals); however, we both visualized via data mechanics and know intuitively that in this physically constrained system any cow moving forward in the queue must be countered with other cows being forced backwards and vice versa. If this effect extends beyond isolated fluctuations in daily formation of the queue, then the presence of some animals in the herd might systematically dampen or even completely prevent other animals from demonstrating behavioral patterns that they would otherwise display independently or in another herd with a different social composition (negative interclass correlation between animals). This would not only serve to confound the behavioral mechanisms at play, but such cows whose behaviors are suppressed by their herd mates cannot be said to be contributing fully to the model, potentially reducing the effective sample size. This could allow sampling fluctuations to produce misleading statistical inferences, even in this large sample of animals (41–43).

UML algorithms cannot recover information about behaviors that were never expressed, and so are also not immune to the biasing effects of non-independence between animals. These tools can, however, provide model-free tests of association that may serve as a sanity check for statistical inferences when degrees of freedom may be uncertain. We explore this option here by again combining modern clustering tools with a flexible information theoretic approach to pattern detection (35). First, independent clustering tress were used to subdivide the herd based on queuing records and each of the cow attributed variable. The resulting categorical variables were then used to form contingency tables between queue subgroups and each of the candidate predictor variables. If no relationship existed between these two axes, then a cow belonging to a given row category based on queue records would be just as likely to belong to any of the column categories based on cow attribute and vice versa. If instead an underlying biological mechanism was present linking these axes, then cows within a range of cow attribute values would be spread unevenly among queue subgroups. Such heterogeneity in cell counts was quantified by calculating a weighted mutual conditional entropy (MCE) value across first the rows and then the columns of the contingency table and averaging the results, which reflected the amount of mutual information shared between the two variables. To determine if the observed MCE value was significantly smaller than would be expected from random fluctuations in the sample, row and column classifiers were randomly permuted across cows to remove any underlying bivariate relationship and MCE recalculated. This randomization procedure was repeated over 2,000 iterations, and the observed entropy value compared to the resulting empirical CDF to produce a $p$-value for the significance of the bivariate association. Mutual conditional entropy tests were performed for all significant or marginally significant linear effects for both regression models. While the number of clusters used to discretize the cow attribute and queue records may be specified *a priori* provided strong biological reasoning or empirical evidence, mutual conditional entropy tests were here preformed on a grid from two clusters up to the highest visible granularity of the corresponding clustering tree, and the optimal metaparameter values selected by minimizing the average marginal rank.

## Exploring Associations Between Sensor and Queue Records

Previous studies seeking to identify factors that predict an animal's parlor entry position have focused primarily on biological drivers of queueing behavior related to productivity, health, and traditional measures of fitness such as age and size (44). As this herd was also fitted with ear tag accelerometers, it is here also possible to explore relationships between queue position and behavioral patterns displayed between milkings. Due to the size of these datasets, however, this small step beyond the bounds of the existing literature constitutes a considerable leap in statistical complexity within a linear modeling framework. A multivariate mixed model that considers all observations from either dataset would exceed the capacity of many solvers (45). A simpler approach to exploring this relationship might therefore be to compress the information available in parlor entry records into a grouping variable and then attempt to identify differences in the various home pen behaviors across the resulting subsections of the herd.

We implement this strategy here by using the *nlme* package to fit linear mixed models, with cow fit as a random intercept, against each of the five behaviors recorded by the CowManager platform (non-activity, activity, high activity, rumination, eating) and also average body temperature (40). To avoid the risk of anomalous behaviors that might skew model inferences, only cows with no recorded health events were used. Hour of the day was fit as a categorical variable to capture cyclical patterns. Days on trial was also fit as a categorical fixed effect to allow for non-smooth longitudinal changes in behaviors due weather and also the shift to pasture. Finally, queue groups were determined by arbitrarily dividing the herd into quartiles based on median entry position. The resulting categorical variable was then fit as both a main effect and an interaction effect against both cyclic and longitudinal time effects. Due to the size of the model, temporal correlation and heterogeneous variance models both exceeded

the capacity of this package to converge. Comparisons of the cyclic and longitudinal trends in behavioral patterns between queue groups were made using the plotting utility available in the *emmeans* package (46), with the complete results provided in the **Supplemental Materials**.

While linear models provide an expedient means to statistically evaluate targeted experimental hypotheses, the more open-ended approach to knowledge discovery provided by UML algorithms may offer an advantage in exploratory data analysis problems such as this. We explore the utility of this alternative strategy here by again employing a mutual conditional entropy (MCE) test to identify significant associations between these two behavioral axes (35). The flexibility of hierarchical clustering tools allows this technique to be directly extended from the previous section, which compared repeated measures of queue position against a univariate covariate, to accommodate both high dimensional datasets. For each parameter recorded by the CowManager platform, this model free test of association was performed on the complete sensor record, on subsets of the records corresponding to each of the three lounging periods (morning, afternoon, and night), and finally on a subset of the records where observations from all three lounging periods had been aggregated. As in the previous section, the number of clusters used to discretize queue and sensor data were evaluated on a grid, here from tree depths 2–10. To characterize the divergent behavioral patterns across queue groups identified by significant tests of association, tube plots were created by plotting each within-day subgroup median on a circular grid and then stacking rings to form a tube using the 3D plotting tools in the *plotly* package (28).

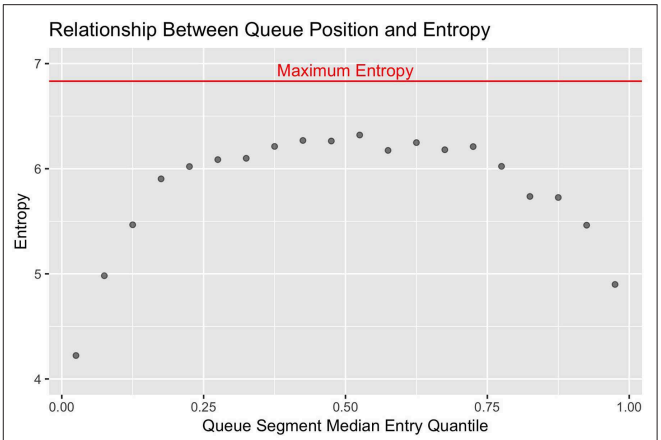


**FIGURE 1 |** Entropy estimates from observed (red) and randomly permuted (blue) datasets are plotted against the median quantile value for the corresponding segment of the queue. The front and end of the queue are the least stochastic, but all sections of the queue demonstrate lower entropy than with purely random queue formation.

# RESULTS AND DISCUSSION

## Quantifying Degree of Randomness

Looking first at the entropy calculations for each segment of the queue visualized in **Figure 1**, it is clear that all parlor entry positions are not stochastically equivalent. The same animals are seen consistently at the very front and back of the queue, such that the resulting entropy values are far lower than would be seen with a purely random queueing process. Moving toward the middle of the queue, however, there is progressively less consistency in the animals present across milkings, such that the observed entropy values approach a random process. Looking next at the stochasticity demonstrated by each individual cow in **Figure 2**, we see there is again a clear gradient. Cows with median entry quantiles at the front and rear of the herd again show far greater consistency in their entry positions. As their median quantile position moves toward the center of the herd they become more variable in their entry positions over the observation window. This gradient is seen using both entropy and variance as estimators of stochasticity, but is more visually distinct using entropy estimates. While discretizing an intrinsically continuous parameter results in a loss of information, we see here that this sacrifice has excluded extraneous noise in the system to bring the underlying stochastic pattern into clearer resolution. This data thus highlights the potential upside of amending entropy estimates to the traditional cadre of summary statistics, particularly when working with outcome variables that are prone to extreme or anomalous values.

In examining the results of the permutation tests, nearly all animals demonstrated significantly less stochasticity in their entry positions at the standard $\alpha = 0.05$ significance level as compared with a completely randomized queueing process. Only 3 cows out of 114 overlapped with the empirical distribution of entropy estimates under a randomized queueing pattern, and only 1 cow overlapped when variance was used as the estimator of stochasticity. This suggests that nearly all animals in the herd might contribute some information about the underlying non-random patterns in queue formation to subsequent analyses; however, the amount of information they contribute may not be equal, as there is considerable heterogeneity between cows. Of greater concern, this heterogeneity is systematic, as there are no cows showing high consistency in entry quantile in the center of the queue. If this pattern is not driven by variability in the underlying predictors of queue position, but instead reflects either an underlying behavioral mechanism or something even more fundamental to this system such as the inherent domain constraint (18), this could lead to inaccurate statistical inferences. To avoid such risks, these simple visualizations provide clear evidence that a non-trivial variance model should be incorporated into the model specification phase to accommodate the heterogeneous variance structures in this dataset.

Finally, the insights gleaned from these entropy-based visualization techniques agree well with the prior literature. Previous studies have repeatedly determined milk order records to be significantly more consistent than would be expected from a random queuing process using an array of correlation and regression-based approaches (10, 12, 16–18, 47). Fewer papers, however, have explored differences in the consistency of entry positions between animals. Gadbury (13) observed that only

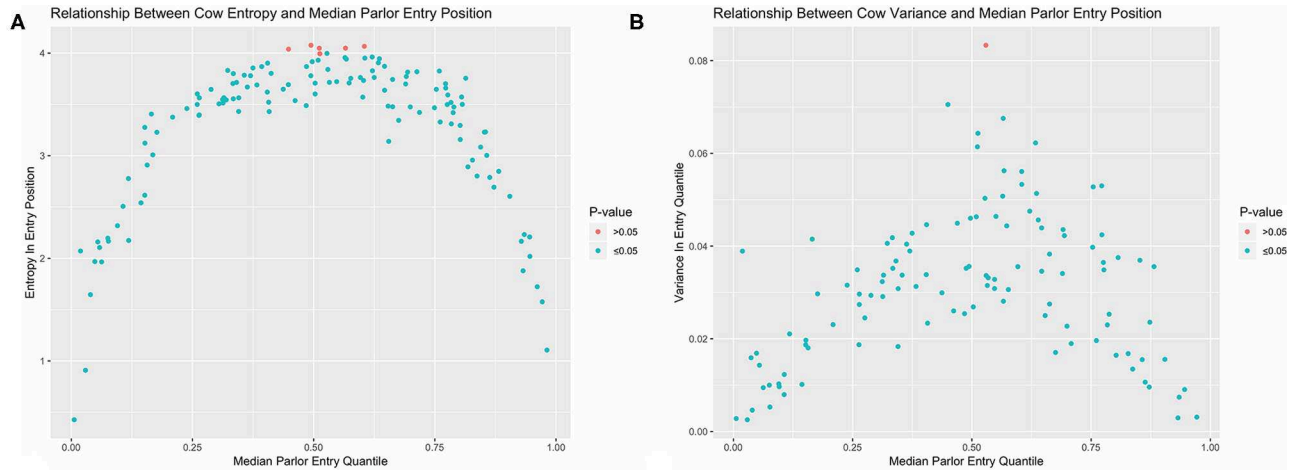


**FIGURE 2 |** Observed entropy **(A)** and variance **(B)** values for each cow plotted against their corresponding median entry position. Cows with the greatest consistency in parlor entry position are found at the front and end of the queue. With both estimators, nearly all cows in the herd demonstrate less stochasticity in parlor entry position than completely random queue formation as determined by permutation test.

a subset of his herd seemed to demonstrate clear preferences for parlor entry positions. Such preferences do not appear to have been constrained to the front or back of the queue, however, as Gadbury (13) also reported animals with a preference for the middle of the queue. In a more recent analysis with large commercial herds, however, Beggs et al. (18) reported a nearly identical parabolic relationship between mean entry quantile and variance. With clear and consistent evidence of non-random patterns having been recovered from this dataset, further investigation of the behavioral mechanisms that might give rise to such heterogeneity in milk order records was clearly warranted.

## Visualization of Inter-animal Relationships

Visual inspection of the scree plot produced from PCA analysis revealed only one significant dimension was recovered from the original 80-dimensional dataset. To visualize the resulting projections, the first two principal components were plotted (**Figure 3A**). Cows appeared evenly spaced along the first principal axis with no clear gaps between observations. In two dimensions points also appeared randomly scattered with no clear clustering. Thus, the PCA results revealed no compelling visual evidence of social cohesion. The color encoding further revealed that the first principal component conveyed information about the center of each cow's entry quantile observations. As this was the only significant dimension, this may suggest that a linear model to predict variations in central moment would be a reasonable representation of this dataset. This feature of the dataset was not, however, self-evident in the geometric relationships between data points revealed by the PCA projection, and thus might have been overlooked without specification of color encoding by median entry quantile value *a priori*.

Evaluation of eigenvalues returned by the diffusion map embedding identified five significant dimensions. The 3D visualizations of these axes in **Figure 3B** and provided in **Supplemental Materials** revealed quite clearly the underlying linear geometry of this dataset. Color encodings showed that the relative positions of animals along this narrow geometric band were determined by median entry quantile, further reinforcing that central moment was the most defining feature of this dataset. As with the PCA results, cows appeared fairly evenly spread along this linear object, with no clear clustering to suggest social cohesion amongst large or temporally persistent subgroups. Comparing these results with the embedding of the permutated queue records (**Figure 3C**), no clear geometric features were recovered from data simulated from a purely random queueing strategy. This reinforced that the linearity of the observed records was not simply an artifact of the physical linearization of cows as they enter the parlor single-file, but a reflection of a consistent pattern in queue formation that might be driven by some underlying behavioral or biological mechanism.

While the diffusion map embeddings convey a clear linear geometry, there was also unexplained curvature in the band along which cows were projected. This proved not to be an inherent feature of the data itself but a harmonic artifact imposed by the spectral value decomposition of the graph Laplacian used to deduce the shape of the underlying network between cows (48). Such a mathematical operation has several physical interpretations. One is that an singular value decomposition (SVD) of the Laplacian is akin to walking around an object in the dark and striking with a mallet at many points across its surface so that the quality of the resulting sounds can be used to discern its shape (49). The linear geometry of this dataset forms a "rope-like" network (48). When the SVD decomposition "strikes" such a network to assess the quality of sound produced, it responds like a plucked guitar string. As a result, each axis of the subsequent embedding contains an element of the harmonic series, producing the curvature seen in these milk order visualizations. Fortunately, this artifact can be described by closed form equations (48) and imposed onto

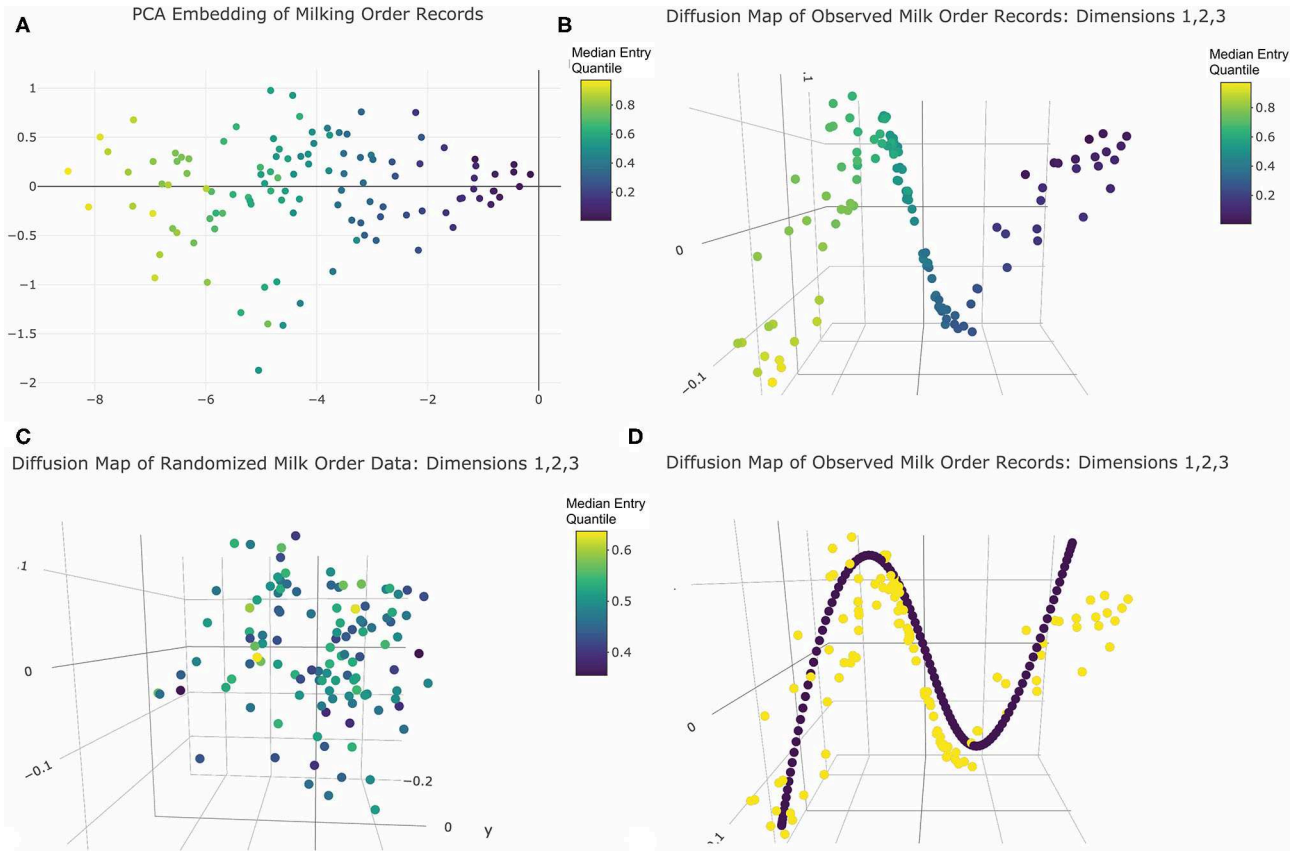


**FIGURE 3 |** Dimension reduction of milk order records colored by median entry quantile value: **(A)** PCA embedding returns one principal axis reflecting each cows central moment of entry position. **(B)** The first three significant axes of diffusion map embeddings reveals a clearly linear underlying geometry reflecting central moment. **(C)** Diffusion map embeddding of data from the simulation of purely random queue formation recovers no distinctive geometry. **(D)** Harmonic series (purple points) imposed over observed cow records (yellow points) reveal curvilinear geometry is a embeding artifact. Note: Interactive versions of 3D plots are availble in **Supplemental Materials**.

the data to aid in discerning authentic geometric features of the data (**Figure 3D**). Thus, while diffusion map did provide a clearer geometric representation of the inherent linearity of this dataset than PCA, this dataset al.so reinforces that modern manifold learning techniques are also not infallible in recovering the underlying geometry of high dimensional data. While such embedding techniques may provide helpful insights into the underlying structure of large datasets, a conservative approach to visual interpretation of such results is still warranted.

## Characterization of Temporal Dynamics

Independent visualization of parlor entry records from each individual cow (see **Supplemental Materials**) revealed that the majority of animals in this sample were surprisingly stationary in their queueing position. Animals that frequented the front and end of the queue, being more consistent in their entry position, provided clearer visual evidence for a lack of temporal trend. Cows in the middle of the queue showed far greater variability in their entry positions, making it more difficult to visually discern temporal trend from stochastic fluctuations. Only two animals were identified as having a clearly visible trend: cow 13,467, who had no recorded health events, and cow 13,826, who was diagnosed with metritis during the enrollment phase early in the trial. Both cows showed similar trajectories, starting nearer the end of the herd and moving progressively forward toward the front, but neither change in queue position coincided with the shift to overnight pasture access.

This consistency in queue position was further reflected in a clear linear association between median entry quantiles from overnight pen and pasture subperiods (see **Figure 4**). A slightly wider spread was discernable amongst cows occupying the middle ranks, but for the majority of animals, median entry quantile values did not change more than ±0.2. Among the handful of animals demonstrating a more extreme shift, these jumps tended to be in the forward direction toward the head of the queue. Overall, fewer extreme shifts were seen in this dataset than in a similar bivariate means plot provided in Beggs et al. (18), though this may simply be a reflection of the longer subperiods over which median entry positions were assessed. Correlations between these values were also quite high, with a Pearson correlation estimate of 0.91 ($p < 2.2e\text{-}16$) and a Kendal Tau estimate of 0.74 ($p < 2.2e\text{-}16$). These values are, as

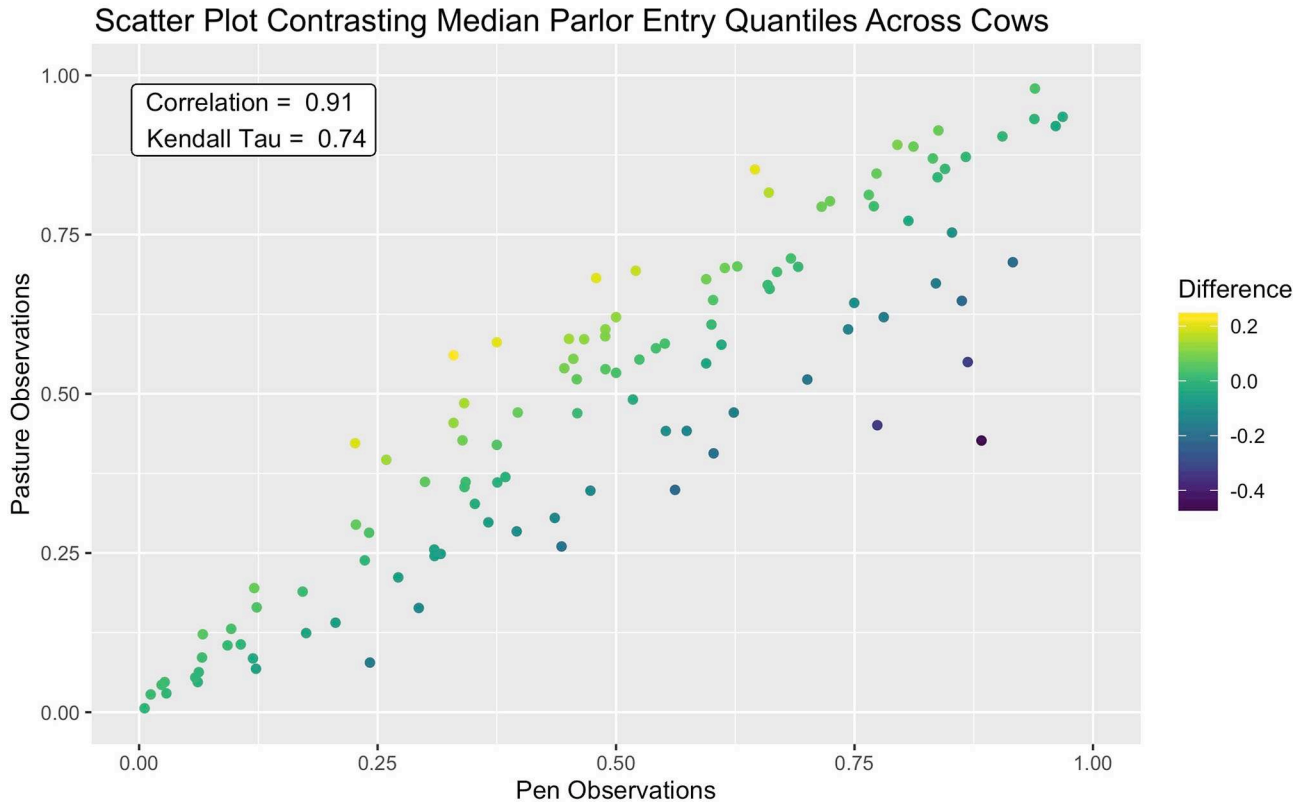


**FIGURE 4 |** Scatter plot showing strong agreement of median parlor entry quantiles between the pen and pasture subperiods. A small portion of cows which demonstrated a larger shift in entry position moved forward in the queue after the shift to pasture from pen.

expected, higher than the estimates of consistency reported for individual milk order samples (17, 19), but on par with results using subperiod averages on similar time scales (12, 16, 18). Given the extreme shift in management routine spanning these two subperiods, however, this level of stability in parlor entry positions was an unexpected result. Such resilience to changes in overnight housing environment and the subsequent distance traversed to access the parlor could suggest that milking order is largely determined in the crowd pen, a result supported by early observations by Soffie et al. (12), who reported little correlation between the order of cows exiting the home pen and entering the parlor past the first few animals.

Collective assessment of entry quantile records using data mechanics visualizations did, however, reveal additional temporal features not identified using independent visualizations of cow records or collective assessment of aggregate records. The first and perhaps must surprising insight was that, with finer granularity in number clusters applied to the temporal (row) axis, data mechanics identified several days with anomalous queuing patterns. In **Figure 5**, a total of 8 column clusters are imposed without any social stratification on the subset of cows with no health events. If these records were completely stationary with no temporal effects, we would expect days to be randomly partitioned into these eight categories. Instead 4 days are isolated from the remaining observations. Days 85 and 91 are separated into clusters of size $n = 1$, and 89 and 91 are also isolated into their own cluster of size $n = 2$. Looking from left to right along the heat map to identify temporal heterogeneity, it is easy to see that on these observation days animals typically occupying the extremes of the queue appear to have been pushed toward the center and animals typically found in the center of the herd were either pushed toward the extremes or inverted their tendency to stay toward the front or end of this middle section of the queue. While some of the entry quantile values encompassed by these observation days would likely be identified as outliers for individual cows, other values would likely be deemed irregular but not worthy of exclusion. These clustering results, on the other hand, suggest that either transient environmental or internal social factors have disrupted the entire herd and caused them to collectively respond with highly irregular queuing patterns. As the row axis is stratified to allow for non-homogeneous temporal responses across subsets of animals, these same days are consistently isolated from the remainder of the dataset, reinforcing that these observations constitute an outlier that should be excluded from any downstream analyses.

Looking next at the coarser stratifications of the temporal axis, we also see that pen and pasture observations are not equally dispersed among the column clusters. As the animal (row) axis is more finely stratified to allow for social heterogeneity within the herd, the source of the temporal heterogeneity between these

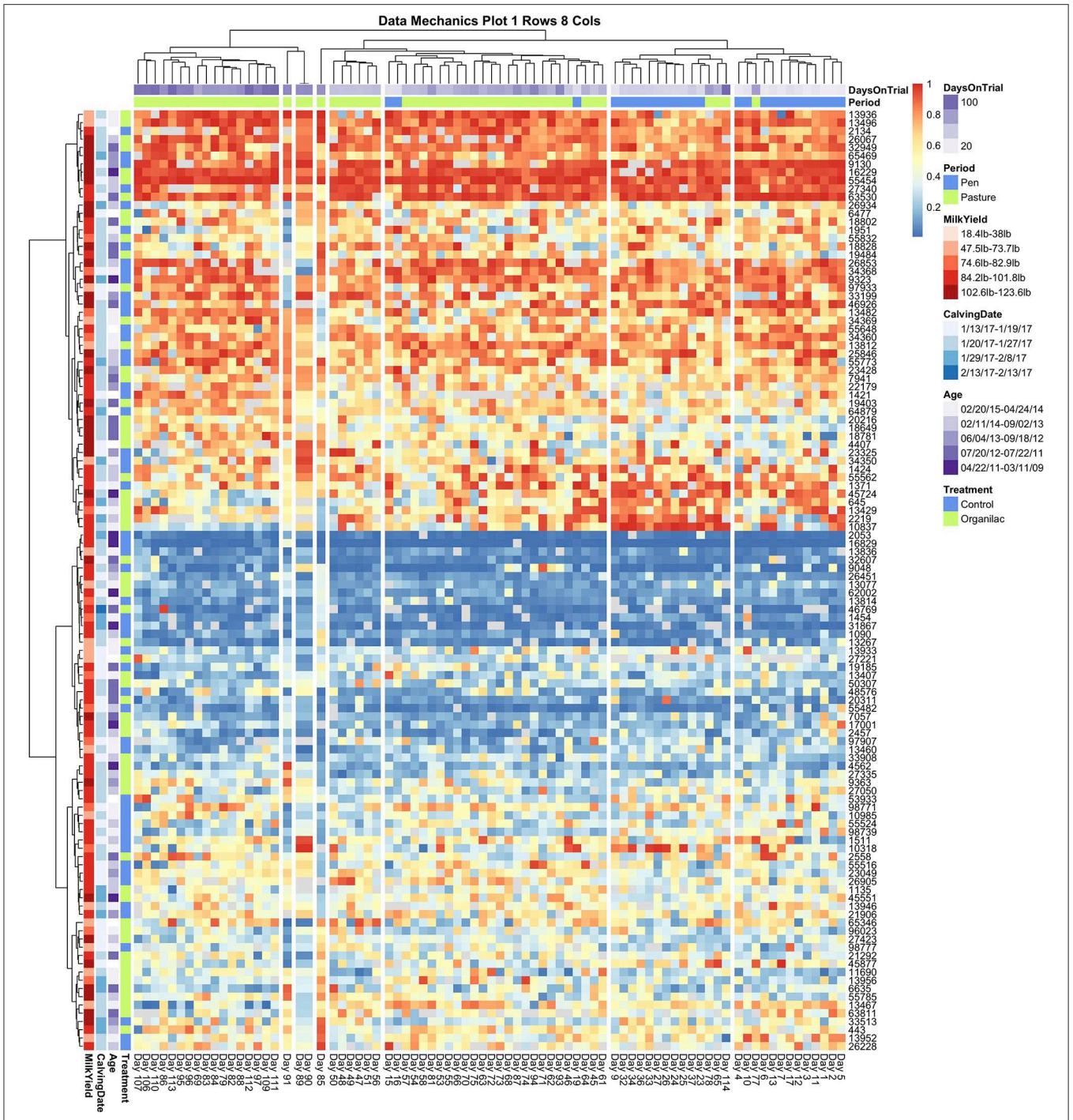


**FIGURE 5 |** Data Mechanics visualization of cows with no recorded health events. Clustering along the temporal axis has isolated 4 days of milk order observations in middle of the pasture subperiod with anomalous queuing patterns, which can be viewed as irregularities in color values scanning from left to right. These days remain isolated in Data Mechanics mappings which also allow for social stratification along the row axis (see **Supplemental Materials**), suggesting that these observations likely constitute outliers. Color annotations along the column axis reveal observations from the pen and pasture subperiods remain fairly distinct. No clear patterns or gradients are seen on the row color annotations for cow attributes, even though the heat map itself clearly reflects a gradient along the column axis driven by individual differences in queue entry position. Progressive clustering of the row axis did not bring any additional patterns in cow attribute variables into clear resolution.

two environments comes into resolution. In **Figure 6**, which contains entry quantile observations on both sick and healthy animals, pen and pasture observations are perfectly stratified across only two column clusters. Looking at the subsets of animals who consistently entered at the front and rear of the herd, entry quantile values appear quite homogenous in color

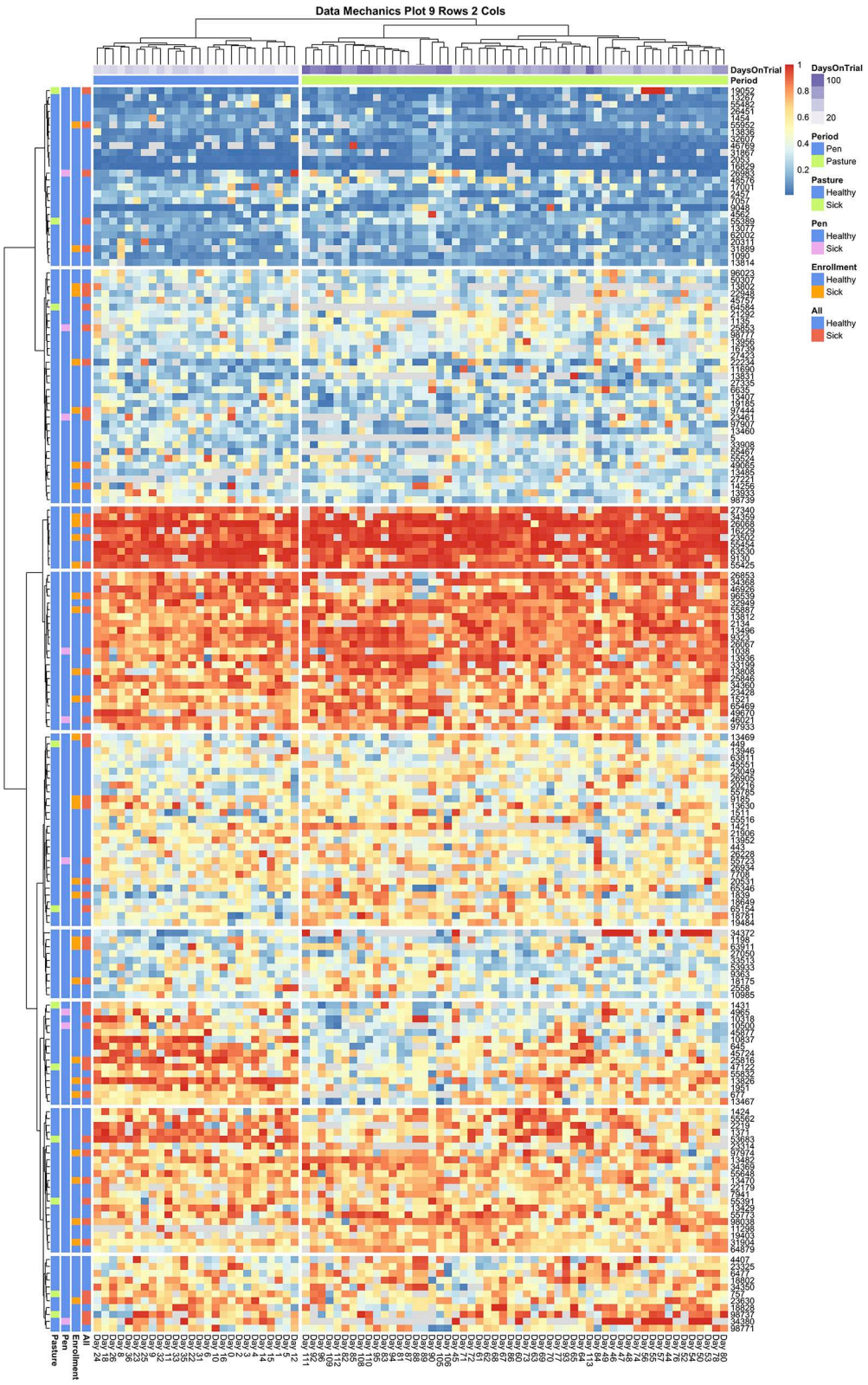


**FIGURE 6 |** Data Mechanics visualization of all cows. Color annotations of the column axis reveal that coarser clustering along the temporal axis has revealed a perfect division of observations between pen and pasture subperiods. Scanning from left to right within the heat map, cows occupying the front and rear of the queue appear consistent in entry quantile values. Cows in the center of the herd appear to be the source of this temporal heterogeneity, as evidence by systematic changes

*(Continued)*

**FIGURE 6 |** in color along the column axis. In the cluster of animals starting with cow 1431, there is a progressive shift from the rear of the herd in the pen period toward the front of the herd in the pasture. As row color annotations reveal not all these animals have recorded health events on record, this pattern likely cannot be explained away by anomalous behaviors from acute or chronic illness.

between the two temporal clusters. Scanning from left to right among the subgroups of animals that frequented the center of the queue, on the other hand, systematic fluctuations in daily entry quantile values can be seen even without finer temporal stratification. This pattern is clearest in the cluster which contains both cow 13,467 and cow 13,826—the two animals identified by independent inspection of cow entry quantile plots to show evidence of non-stationarity. In this subgroup, cows showed a tendency to frequent the latter half of the queue when coming to the milking parlor from the home pen, but during the pasture period showed progressively greater proclivity to enter in the front half of the queue. Where this shift is the most uniform in the latter half of the pasture period, we also see a compensatory pattern in the subgroup directly above, where cows shifted from nearer the front to the back half of the queue. Whether these results reflect the coordinated movement of relatively small social subgroups or just a common response to environmental conditions is impossible to say from this data alone. These results do make it clear, however, that not only are the cows occupying the center of the queue less consistently in their entry position, they are also less stable in their entry pattern. Further, these visualizations underscore that these divergent dynamics in the pen and pasture subperiods cannot be captured by a simple fixed effect term. The simplest option would be to drop from the analysis the animals that show the strongest non-stationary patterns. With such a large group, this would still leave ample observations to maintain statistical power, but could risk biasing the subsequent inferences. Alternatively, by specifying a heterogeneous variance model between animals, as was deemed necessary in the original entropy plots, the influence of these cows on the fitted model may be reduced sufficiently that deviations from the assumption of stationarity in this subgroup might not unduly destabilize the final model.

Finally, some preliminary insights can be gleaned from the cow attributes added to the row margins of both heat maps. In **Figure 6**, animals with documented health events appear fairly evenly dispersed across subsections of the queue. A slightly lower rate of illness might be attributed to animals that consistently occupied the very front of the queue, and perhaps a marginally higher rate of transition diseases was seen in the animals at the very rear of the queue, but these patterns appear subtle at best and thus likely not the only determinant of queue position. This result was somewhat surprising, as previous research has suggested that sick animals tend to populate the rear of the queue (11, 16, 17, 19). If this previously reported trend is driven by a reluctance among animals in the acute phases of a disease to move, it is possible that the daily health checks prescribed in this experimental trial succeeded in identifying and removing sick animals from the herd sufficiently early that this behavioral mechanism was not at play in this dataset. This might suggest that inclusion of these additional animals into subsequent analyses might not unduly bias subsequent behavioral inferences. Of perhaps greater concern to subsequent modeling is the lack of clear color gradients among cows attribute values across the queue, which could indicate that underlying associations may either be weak or that there are complex interaction effects creating a non-uniform trend.

## Linear Analysis of Cow Attributes

For both the full dataset and the subset of healthy animals, likelihood ratio tests revealed the heterogeneous variance model allowing for differing degrees of variability in queue position across cows to be a costly but necessary model component ($p < 0.0001$). With the model fit to cows with no recorded health events, significant linear associations were recovered for two fixed effects. Cows with higher peak milk yields demonstrated a tendency to enter nearer the rear of the queue ($\hat{B} = 0.14$, $F_{1,96} = 9.58$, $p = 0.003$). A significant interaction term revealed this trend was further amplified for older cows ($\hat{B} = 0.07$, $F_{1,96} = 6.11$, $p = 0.015$). No other terms approached significance for this dataset. With the model fit to all cows that attended at least 50% of recorded milkings, no predictors were significant at the $\alpha = 0.05$ cutoff. Peak yield remained marginally significant ($\hat{B} = 0.06$, $F_{1,152} = 2.93$, $p = 0.089$), as did the interaction term between peak yield and cow age ($\hat{B} = 0.04$, $F_{1,152} = 2.92$, $p = 0.090$). With this larger dataset, however, cow age also demonstrated a marginally significant trend, indicating older cows tended to be nearer the front of the queue ($\hat{B} = -0.07$, $F_{1,152} = 3.84$, $p = 0.052$).

In contrasting the results of these two models, the loss of significant association between entry quantile values and peak yield with the addition of sick animals is perhaps not surprising. If a disease challenge early in the trial curtailed peak lactation in these cows but did not cause chronically deficient production, then the 95th quantile value of milk yield used here to estimate peak lactation level may not adequately reflected the overall productivity of these animals across the duration of this extended trial, obscuring the underlying biological mechanism. The emergence of a nearly significant association between entry quantile and age with the addition sick animals, however, is more difficult to explain. Given that peak yield and age are highly correlated biological parameters ($r_{all} = 0.66$, $r_{healthy} = 0.70$), this sample may simply contain too few older cows with low productivity levels by which to disentangle the positive association with peak yield from the negative association with cow age. Alternatively, if a diseased state permanently alters a cow's queueing pattern and if risk of health complications in turn varies with age, then health status may be a lurking variable masquerading as an age effect. In either case, a relatively small number of animals may be unduly influencing statistical inferences.

Visual examination of predicted queue positions plotted against age and peak yield for both the full dataset (see **Figure 7**) and healthy subset (see **Supplemental Materials**) seem to confirm these misgivings. Looking first at age, the first lactation heifers, being evenly spread across the center of the queue, cannot be driving this linear effect. Among the multiparous animals, the five cows seen consistently in the front of the queue are indeed among the oldest in the herd, but if this handful of animals and their corresponding queue positions are ignored, a clear gradient is not visible among the remaining cows. Results of the mutual conditional entropy tests confirm this suspicion. For the disease free subset the MCE test confirms the insignificant association found in the linear model ($p_{2,2} = 0.103$). For the full dataset, where the linear effect is marginally significant, the MCE test does not ($p_{7,2} = 0.305$). This suggests that either that age effect is only discernable after adjusting for peak yield or that the association is not robust.

Looking next at peak yield, a clear global trend could not be discerned. Among the lower-yielding cows, a group comprised predominantly of heifers, a linear trend is difficult to discern, but amongst older cows a slight positive gradient is perhaps perceptible. Results of the mutual conditional entropy tests not only confirmed this trend among the healthy animals($p_{3,5} = 0.036$), but also within the full dataset ($p_{2,3} = 0.012$) where the linear effect was only suggestive. Visualization of the contingency table for this later result revealed no distinctive pattern among the lowest and highest yielding clusters, but a nearly perfect division of roughly 50 moderate-yielding cows into the leading queue cluster. This result suggests that the MCE tests may also be used in mixed modeling analyses to recover non-random patterns that are not well-represented by linear trends. Such a non-linear trend here could belie more complex interaction effects between these or other unmeasured biological drivers of queue position. Alternatively, a multilevel model may be necessary to disentangle complex hierarchical relationships between the drivers of position preference and a cow's ability to assert that preference.

Contextualizing these results within the existing base of literature underscores the inconsistency in drivers of queuing behaviors. With respect to milk yield, several studies have found no significant association (12, 16), but among those that have, most have reported high yielding cows frequent the front of the queue. Differences in motivation to obtain feed might explain this result. In early studies, cows were offered concentrate in the milking parlor, which may have increased the motivation of high yielding animals with greater energy deficiencies to enter the parlor (11, 13). In more recent work, cows may have been motivated to access limited feed bunk space on commercial dairies (19) or to obtain prime pasture (50). In this study, as all animals were locked following milking to facilitate feeding treatments and health checks, cows would have had ample access silage regardless of queue position. Alternatively, Rathore (11) suggested greater intermammary pressure might motivate high yielding animals to be milked earlier. As this herd was milked three times daily, however, this biological driver may also have been attenuated. Indeed, among modern studies with herds milked thrice daily, Polikarpus et al. (16) found no significant correlation and Grasso et al. (17) also found high yielding cows frequented the rear of the queue. Ultimately, as yield is influenced by a wide range of health and management factors, any number of confounding variables might be implicated in explaining this somewhat unexpected result. In this study a significant linear association between age and entry position was not found. Recent work by Berry et al. (19), which identified a non-linear trend across parity, and by Grasso et al. (17), which highlighting significant interactions of parity with other biological drivers of queue position, suggests that a linear effect may not adequately capture the underlying biological relationship. A larger and more structured sample may be necessary to bring more complex age dynamics into clearer resolution.

## Exploring Associations Between Sensor and Queue Records

Visual inspection of means plots produced from mixed model analysis of sensor records recovered only a handful of statistically significant differences between queue quartiles when hour and day effects were assessed individually, but several global trends were still readily visible. With respect to minutes recorded as active, the 1st−3rd queue quartiles were visually indistinguishable in their cyclical behavioral patterns, but cows in the fourth queue quartile were consistently more active, particularly during the night and morning lounging period. With respect to longitudinal trends across days, fourth queue quartile animals were again more active across the observation window, whereas cows in the first queue quartile were consistently the least active. These patterns were somewhat mirrored in the longitudinal and cyclical analysis of high activity minutes, but the pattern was both less distinct and less consistent. No clear qualitative insights could be drawn for cyclical or longitudinal patterns in non-activity. Cyclical patterns in minutes spent eating were not seen overnight or in the afternoon, but first queue quantile cows may have spent slightly more time eating after the morning milking. Longitudinal analysis of eating patterns suggested cows in the fourth queue quartile spent relatively less time eating, whereas the cows in the first and second queue quartile consistently spent more time at the bunk. This contrasted with longitudinal results for minutes spent ruminating, where the cows in the second queue quartile were consistently low. No clear distinctions between groups were recovered in cyclical rumination patterns. Temperature patterns were, surprisingly, the most visually distinct of all the sensor parameters. Cows in the first queue quartile were consistently lower in body temperature in both the longitudinal and cyclical time dimensions as compared with the remainder of the herd.

While the preceding analyses revealed few statistically significant differences at individual time points, collective analysis of days and subsets of the 24 h management cycle would uundoubtedly return statistically significant differences for the broader qualitative trends visually identified via mean plots. Within a linear modeling framework, however, this constitutes no small task. For all of the above models, Wald's tests revealed Group-by-Hour interactions effects to be highly

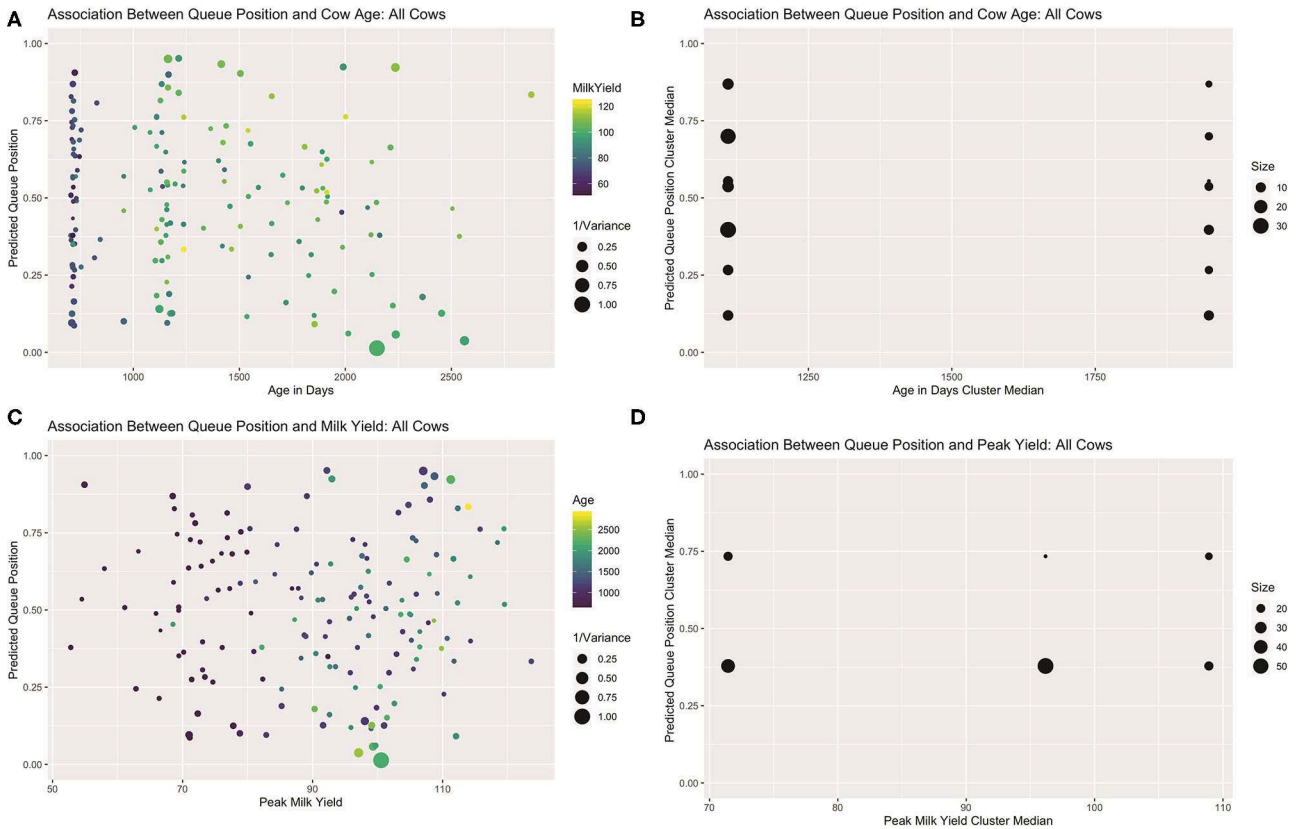


**FIGURE 7 |** Visualizations for all cows that attended >50% of observed milkings. Scatter plots of predicted queue position against cow age **(A)** and peak yield **(B)**, scaled for heterogeneity in repeated samples of entry quantiles. For both attributes, linear trends are only readily discerned among multiparous animals. Sparsity in parity 3+ animals might skew the calculation of age effect as a continuous variable. **(B)** Visualization of contingency table results derived from independent clusterings used in MCE test of bivariate association. Age effect is lost with empirical discretization **(C)**. A non-linear trend is recovered with peak yield **(D)**.

significant components of the model ($p < 0.0001$). Group-by-date interaction effects were also significant for activity, high activity, and temperature models ($p < 0.05$). This suggests that these models should not be simplified to a single cyclical or longitudinal trend, which would allow overall differences between groups to be tested via a single group intercept term. Targeted hypotheses comparing comprehensive trends between groups would instead require formulation of linear contrasts—a daunting task with so many fixed effects terms used to accommodate the high sampling frequency and extended observation period of this dataset. Further, as with the linear models with cow attributes, behavioral synchronization due to social cohesion or compensatory use of physical resources in the pen could again create non-independence between animals in such sensor records. Any such issues in estimation of model degrees of freedom, compounded with the inability to fit behaviorally and empirically compelling correlation and variance models, would only serve to further confound the estimation of appropriate $p$-values from these models.

Fortunately, the qualitative trends identified via the preceding means plots largely aligned with the significant bivariate associations identified by mutual conditional entropy tests summarized in **Table 2**. Activity again proved to be the most distinctive behavioral axis. Significant associations were identified for all three lounging periods when analyzed both independently and in aggregate, with the afternoon lounging periods being the most distinct. High activity also showed a significant relation to queue records, but this association may have been driven predominantly by overnight lounging period. Whereas, no clear qualitative patterns were identified for non-activity data via the means plots, a significant association with queue records was identified during the afternoon lounging period. A highly significant relationship was identified for time spent eating for the full sensor record, but given that time budgets recorded by this platform were segmented somewhat arbitrarily at the start of each hour, this result may simply reflect a lag in the arrival of cows to the feed bunk after exiting the parlor. Significant associations were not found during the lounging periods at the standard $\alpha = 0.05$ cutoff, though records from the afternoon lounging period approached significance. These results were mirrored in rumination patterns, where again no significant association was recovered, but the afternoon lounging period approached

**TABLE 2 |** *P*-values generated from mutual conditional entropy tests comparing queue records to sensor logs.

| | All | Lounging | Morning | Afternoon | Night |
|---|---|---|---|---|---|
| Non-activity | $<0.001_{7,5}$ | $0.048_{2,5}$ | $0.432_{8,7}$ | $0.002_{2,5}$ | $0.132_{2,6}$ |
| Activity | $<0.001_{3,8}$ | $0.006_{2,8}$ | $0.038_{2,11}$ | $<0.001_{2,3}$ | $0.033_{2,9}$ |
| High Activity | $0.052_{2,9}$ | $0.028_{2,2}$ | $0.272_{10,9}$ | $0.306_{3,4}$ | $0.014_{2,4}$ |
| Eating | $0.004_{7,6}$ | $0.234_{7,5}$ | $0.188_{6,4}$ | $0.066_{2,4}$ | $0.212_{9,2}$ |
| Rumination | $0.021_{2,4}$ | $0.05_{9,3}$ | $0.325_{7,2}$ | $0.083_{10,3}$ | $0.152_{8,10}$ |
| Temperature | $<0.001_{5,10}$ | $0.004_{5,5}$ | $0.022_{7,3}$ | $0.006_{3,5}$ | $0.015_{5,3}$ |

*Subscripts represent the number of clusters used to discretize the row variable (queue records) and column variable (sensor records). For example, in the test for morning non-activity, cows were assigned to 8 clusters using queue records and 7 clusters using sensor logs. Activity and temperature data demonstrated the strongest association with queue records. The afternoon lounging period produced the strongest associations between queue and all sensor dimensions save for high activity, which showed the strongest distinction overnight.*

significance. Finally, as with the linear modeling results, temperature proved highly distinct between queue subgroups for all subperiods.

Visual inspection of tube plots produced with median queue subgroup values again yielded insights comparable to the linear modeling results (**Figure 8**). Based on the results of the MCE tests, all behavioral axes cows were clustered into two subgroups based on queueing records, with Group 1 cows consisting of 80 animals at the front of the queue, and Group 2 cows constituting the 34 animals in the rear. Tube plots of minutes spent active revealed Group 2 cows to be more active across all three lounging periods. This pattern was the most consistent in the morning and overnight lounging periods, though this difference was ultimately quite subtle and seldom constituted more than a few minutes. In the afternoon subperiod there was evidence of several periods with anomalously high activity levels, most of which occurred post-pasture access. The significant association recovered for minutes spent highly active in the overnight subperiod appeared to be largely driven by increased activity immediately following the evening milking, which could reflect divergent home pen behaviors, but might also have been driven by delays in milking. To complement these results for active and high active minutes, the significant association for afternoon non-activity records appears to have been driven by increased non-activity among the Group 1 cows during the 3 h immediately preceding the night milking. As anticipated, differences in time spent eating were largely restricted to the 2–3 h immediately following milking. Cows only lingered at the feed bunk during the morning lounging period, where median eating times for Group 1 cows were perhaps slightly higher. Similarly, differences in rumination also appeared restricted to time periods immediately following milking, with no clear differences seen during the lounging period with this coarse stratification of animals. Finally, as with the mean plots, body temperature values again produced surprisingly distinctive results. More finely segmented into five queue groups by the mutual conditional entropy test, the tube plots proved a slightly cumbersome means of comparing temperature records, but a clear visual distinction could still be made between the Group 2 animals and the remainder of the herd. For all three lounging periods, this relatively small cluster of 17 cows that constituted the very front of the milking queue demonstrated lower median body temperature values, a distinction seen most clearly at night.

The strong agreement between the results of these two analytical pipelines suggests that UML and conventional linear modeling approaches could be used interchangeably or in concert to glean preliminary insights from exploratory analyses of large sensor-based datasets that may inform future hypothesis-driven studies. Perhaps the most surprising result of these analyses, that cows frequenting the back of the queue are consistently more active between milkings, may indeed warrant further exploration. In much of the prior literature, health challenges that impede movement (lameness, subclinical mastitis, etc.) have been identified as the main driver of delayed entry into the parlor (13, 16, 17, 51). In fact, this mechanism is so well-established that it has even been proposed that milk order records might be incorporated into genetic evaluations to improve estimates of health traits (19). As these analyses were run on the subset of animals with no recorded health events, however, it is possible that this dataset has brought other behavioral mechanisms into focus.

One potential explanation for these results might be a dominance gradient. Previous studies have found that animals of low social status frequent the rear of the herd in voluntary movements (10, 52), and social dominance is known to impact resource access in spatially constricted conditions (53, 54) such as those found at the entrance to the milking parlor. If low dominance animals are in turn also forced to wait longer or walk farther to access resources in the home pen, this could potentially explain the increased activity levels of animals found in the rear of the queue. While the early literature has found the relationship between dominance value and milking order to be tenuous at best (11–13, 15), it is possible that such social mechanisms may have been confounded by health status, with linear analyses of limited sample size failing to disentangle these mechanisms in non-disaggregated data. Alternatively, in more recent analyses in automated milking systems, where dominance has proven highly correlated with milking order (55), greater attention has also been paid to "avoiders"—animals that seem to actively avoid social interactions and therefore occupy no clear position in the herd hierarchy (44). On this farm, where resources are not severely restricted and animals are frequently remixed, energetic investments in a dominance hierarchy may offer few returns (2). Such a behavioral strategy might also explain why it is high-yielding multiparous cows and not heifers that occupy the end of the queue. Both these hypotheses are ultimately purely speculative interpretations of these exploratory results; however, if proposals to incorporate milk order records into genetic indices are progressed, any correlations between queueing position and consistent individual differences in home pen behaviors likely warrant closer inspection to mitigate the risk of unintended and potentially deleterious selection pressures.

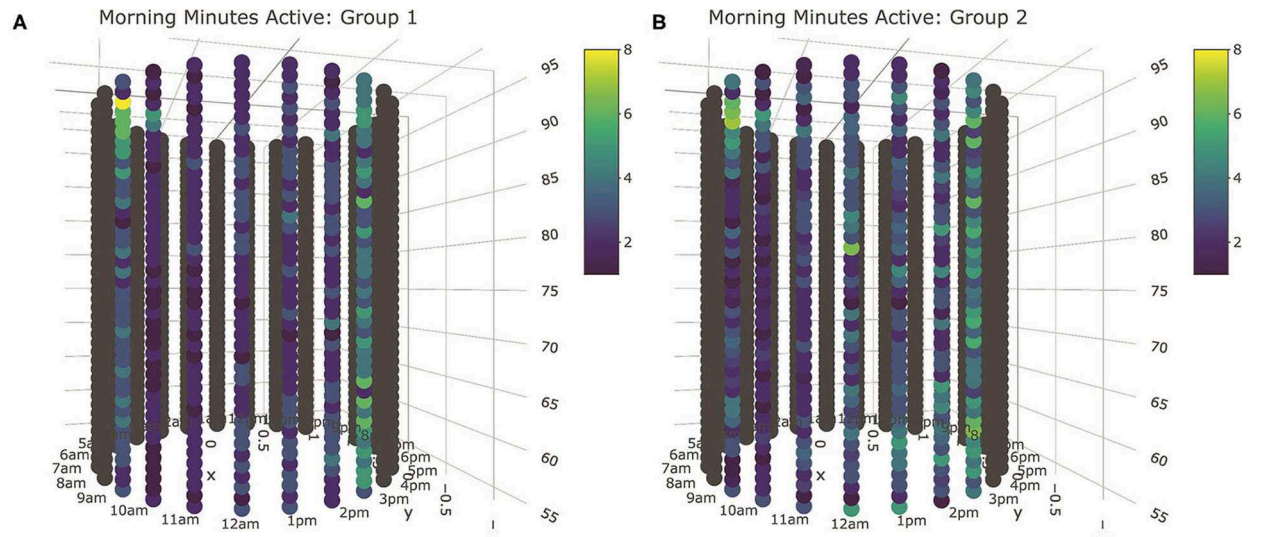


**FIGURE 8 |** Tube plot visualizations for minutes spent active during the morning lounging period. Based on the results of the MCE tests, all behavioral axes cows were clustered into two subgroups based on queueing records, with Group 1 cows **(A)** consisting of 80 animals at the front of the queue, and Group 2 cows **(B)** constituting the 34 animals in the rear. Cyclical patterns can be seen around the diameter of the tube, and longitudinal patterns observed along its length. The median activity level of Group 2 animals, consisting of 34 animals in the rear of the queue, is heightened during the middle of the lounging period for the duration of the trial. Interactive 3D plots for all sensor output fields are provided in **Supplemental Materials**.

## CONCLUSIONS

As with previous studies of milk order records, these analyses perhaps raise more questions than answers. As dairy record management systems grow to accommodate an ever wider range data streams, perhaps future work considering more herds from a wider range of management strategies will succeed in further untangling the complex web of explanatory variables at the individual, herd, and farm levels that drive variation in queueing patterns. This dataset has, none the less, demonstrated the utility of unsupervised machine learning tools in ethological studies using sensor platforms to study larger groups of animals over extended periods of time. While these analyses recovered no evidence of social cohesion amongst large or temporally consistent subgroups, information theoretic approaches succeeded in clarifying the underlying pattern of heterogeneity in error variance between animals and also demonstrated an advantage in recovering evidence of non-uniform patterns in temporal non-stationary over basic EDA tools. After incorporating these insights into the structure of subsequent linear models, these model-free tools then showed some capacity to confirm inferential results where probabilistic assumptions were not strictly met, as well as an aptitude for recovering significant associations not captured by a simple linear effect. This flexible clustering-based approach to identifying significant bivariate associations was then easily extended to accommodate two high dimensional behavioral axes, providing equivalent insights to more computationally taxing mixed effect models. While UML approaches are by no means infallible, as seen here with artifacts produced by the spectral embeddings, these analyses have demonstrated that such tools can add value at every stage of the standard hypothesis-driven linear analysis pipeline, and may even offer an advantages over model-based approaches in early-stage exploratory projects. While many new methodological developments are doubtless on the ethological horizon, we hope this algorithmic toolset will provide a meaningful step forwards to meet the challenges of a future defined by ever larger and more complex data.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Colorado State University IACUC (protocol ID: 16-6704AA). Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

DM and PP contributed to experimental design and data collection. CM and FH contributed to data analysis. CM contributed to manuscript preparation. All authors contributed to manuscript writing and revisions.

## FUNDING

Research support was provided to CM through the National Science Foundation Graduate Research Fellowship and the Deans Distinguished Graduate Fellowship from the UC Davis College of Agriculture and Environmental Science.

## ACKNOWLEDGMENTS

The authors thank Aurora Organic Dairy for facilitating the use of animals and facilities, as well as all the assistance from the farm's personnel throughout the study. The authors would like to thank James Fadel for sharing his expertise in mixed models analysis. The authors would also like to thank Jessica Vandeleest and the McCowan Lab for sharing their experience with Data Mechanics analyses in ethological contexts and Kevin Fuji for contributing sections of the code used to generate these visualizations.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00523/full#supplementary-material

## REFERENCES

1. Dawkins MS. *Observing Animal Behaviour: Design and Analysis of Quantitative Data*. Oxford University Press (2007).
2. Pagel M, Dawkins MS. Peck orders and group size in laying hens: "futures contracts" for non-aggression. *Behav Processes*. (1997) 40:13–25. doi: 10.1016/S0376-6357(96)00761-9
3. Biro PA, Dingemanse NJ. Sampling bias resulting from animal personality. *Trends Ecol Evol*. (2009) 24:66–7. doi: 10.1016/j.tree.2008.11.001
4. Rosser G, Fletcher AG, Maini PK, Baker RE. The effect of sampling rate on observed statistics in a correlated random walk. *J R Soc Interface*. (2013) 10:20130273. doi: 10.1098/rsif.2013.0273
5. Wathes CM, Kristensen HH, Aerts J-M, Berckmans D. Is precision livestock farming an engineer's daydream or nightmare, an animal's friend or foe, and a farmer's panacea or pitfall? *Comput Electr Agric*. (2008) 64:2–10. doi: 10.1016/j.compag.2008.05.005
6. Banhazi TM, Lehr H, Black JL, Crabtree H, Schofield P, Tscharke M, et al. Precision livestock farming: an international review of scientific and commercial aspects. *Int J Agric Biol Eng*. (2012) 5:1–9. doi: 10.3965/j.ijabe.20120503.001
7. Ellen E, van der Sluis M, Siegford J, Guzhva O, Toscano M, Bennewitz J, et al. Review of sensor technologies in animal breeding: phenotyping behaviors of laying hens to select against feather pecking. *Animals*. (2019) 9:1–21. doi: 10.3390/ani9030108
8. Halachmi I, Guarino M. Editorial: precision livestock farming: a 'per animal' approach using advanced monitoring technologies. *Animal*. (2016) 10:1482–3. doi: 10.1017/S1751731116001142
9. Valletta JJ, Torney C, Kings M, Thornton A, Madden J. Applications of machine learning in animal behaviour studies. *Anim Behav*. (2017) 124:203–20. doi: 10.1016/j.anbehav.2016.12.005
10. Kilgour R, Scott TH. Leadership in a herd of dairy cows. *Proc New Zeal Soc Anim Prod*. (1959) 19:36–43.
11. Rathore AK. Order of cow entry at milking and its relationships with milk yield and consistency of the order. *Appl Anim Ethol*. (1982) 8:45–52. doi: 10.1016/0304-3762(82)90131-6
12. Soffié M, Thinès G, De Marneffe G. Relation between milking order and dominance value in a group of dairy cows. *Appl Anim Ethol*. (1976) 2:271–6. doi: 10.1016/0304-3762(76)90060-2
13. Gadbury JC. Some preliminary field observations on the order of entry of cows into herringbone parlours. *Appl Anim Ethol*. (1975) 1:275–81. doi: 10.1016/0304-3762(75)90020-6
14. Reinhardt V. Movement orders and leadership in a semi-wild cattle herd. *Behaviour*. (1982) 83:251–64. doi: 10.1163/156853983X00183
15. Dickson DP, Wieckert DA, Barr GR. Social relationship of dairy cows in a feed lot. *Behaviour*. (1967) 29:195–203. doi: 10.1163/156853967X00118
16. Polikarpus A, Kaart T, Mootse H, De Rosa G, Arney D. Influences of various factors on cows' entrance order into the milking parlour. *Appl Anim Behav Sci*. (2015) 166:20–4. doi: 10.1016/j.applanim.2015.02.016
17. Grasso F, De Rosa G, Napolitano F, Di Francia A, Bordi A. Entrance order and side preference of dairy cows in the milking parlour. *Ital J Anim Sci*. (2007) 6:187–94. doi: 10.4081/ijas.2007.187
18. Beggs DS, Jongman EC, Hemsworth PH, Fisher AD. Short communication: milking order consistency of dairy cows in large Australian herds. *J Dairy Sci*. (2018) 101:603–8. doi: 10.3168/jds.2017-12748
19. Berry DP, McCarthy J. Genetic and non-genetic factors associated with milking order in lactating dairy cows. *Appl Anim Behav Sci*. (2012) 136:15–9. doi: 10.1016/j.applanim.2011.11.012
20. Manriquez D, Chen L, Melendez P, Pinedo P. The effect of an organic rumen-protected fat supplement on performance, metabolic status, and health of dairy cows. *BMC Vet Res*. (2019) 15:450. doi: 10.1186/s12917-019-2199-8
21. Manriquez D, Chen L, Albornoz G, Velez J, Pinedo P. Case study: assessment of human-conditioned sorting behavior in dairy cows in farm research trials. *Prof Anim Sci*. (2018) 34:664–70. doi: 10.15232/pas.2018-01749
22. R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing (2018). Available online at: https://www.R-project.org/ (accessed May 20, 2020).
23. Shannon CE. A mathematical theory of communication. *Bell Syst Tech J*. (1948) 27:379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x
24. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag (2016). Available online at: https://ggplot2.tidyverse.org/ (accessed May 20, 2020).
25. Schein MW, Fohrman MH. Social dominance relationships in a herd of dairy cattle. *Br J Anim Behav*. (1955) 3:45–55. doi: 10.1016/S0950-5601(55)80012-3
26. James G, Witten D, Hastie T, Tibshirani R. *An Introduction to Statistical Learning*, Vol. 103. New York, NY: Springer (2013).
27. Barter E, Gross T. Manifold Cities: social variables of urban areas in the UK. *Proc R Soc A Math Phys Eng Sci*. (2019) 475:20180615. doi: 10.1098/rspa.2018.0615
28. Sievert C. *Plotly for R*. (2018). Available online at: https://plotly-r.com (accessed May 20, 2020).
29. Saul LK, Roweis ST. Think globally, fit locally: unsupervised learning of low dimensional manifolds. *J Mach Learn Res*. (2003) 4:37. doi: 10.1162/153244304322972667
30. Lin B, He X, Ye J. A geometric viewpoint of manifold learning. *Appl Inform*. (2015) 2:1–12. doi: 10.1186/s40535-014-0004-0
31. Kirby M. *Geometric Data Analysis: An Empirical Approach to Dimensionality Reduction and the Study of Patterns*. New York, NY: John Wiley and Sons, Inc. (2001).
32. Izenman AJ. *Modern Multivariate Statistical Techniques*. New York, NY: Springer (2008).
33. Coifman RR, Lafon S, Lee AB, Maggioni M, Nadler B, Warner F, et al. Geometric diffusions as a tool for harmonic analysis and structure definition of data: diffusion maps. *Proc Natl Acad Sci USA*. (2005) 102:7426–31. doi: 10.1073/pnas.0500334102
34. Pinheiro JC, Bates DM. *Mixed-Effects Models in S and S-PLUS*. New York, NY: Springer-Verlag (2000).
35. Hsieh F, Liu S-Y, Hsieh Y-C, McCowan B. From patterned response dependency to structured covariate dependency: entropy

based categorical-pattern-matching. *PLoS ONE*. (2018) 13:1–28. doi: 10.1371/journal.pone.0198253
36. Guan J, Hsieh F. Coupling geometry on binary bipartite networks: hypotheses testing on pattern geometry and nestedness. *Front Appl Math Stat*. (2018) 4:38. doi: 10.3389/fams.2018.00038
37. McCowan B, Beisner B, Bliss-Moreau E, Vandeleest J, Jin J, Hannibal D, et al. Connections matter: social networks and lifespan health in primate translational models. *Front Psychol*. (2016) 7:433. doi: 10.3389/fpsyg.2016.00433
38. Kolde R. *pheatmap: Pretty Heatmaps.* (2019). Available online at: https://CRAN.R-project.org/package=pheatmap (accessed May 20, 2020).
39. Neuwirth E. *RColorBrewer: ColorBrewer Palettes.* (2014). Available online at: https://CRAN.R-project.org/package=RColorBrewer (accessed May 20, 2020).
40. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. *nlme: Linear and Nonlinear Mixed Effects Models.* (2018). Available online at:. https://CRAN.R-project.org/package=nlme (accessed May 20, 2020).
41. Martin P, Bateson P. *Measuring Behavior: An Introductory Guide*, 3rd ed. Cambridge: Cambridge University Press (2007).
42. Forstmeier W, Wagenmakers E-J, Parker TH. Detecting and avoiding likely false-positive findings—a practical guide. *Biol Rev*. (2017) 92:1941–68. doi: 10.1111/brv.12315
43. Bruneel H, Maertens T, Steyaert B, Claeys D, Fiems D, Walraevens J. Analysis of a two-class FCFS queueing system with interclass correlation. In: Al-Begain K, Fiems D, Vincent J.-M, editors. *Analytical and Stochastic Modeling Techniques and Applications.*, Vol. 7314. Berlin; Heidelberg: Springer (2012). pp. 32–46.
44. Danielsson T. *The effect of social rank on milking and feeding behaviour in automatic milking system for dairy cows* (Master's thesis). Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management, Uppsala, Sweden (2004).
45. Snijders TAB, Bosker RJ. *Multilevel Analysis: An Introduction to Basic and Advanced Multilevel Modeling,* 2nd ed. Thousand Oaks, CA: SAGE Publications Ltd (2012).
46. Lenth R. *emmeans: Estimated Marginal Means, Aka Least-Squares Means.* (2019). Available online at: https://CRAN.R-project.org/package=emmeans (accessed May 20, 2020).
47. Dietrich JP, Snyder WW, Meadows CE, Albright JL. Rank order in dairy cows. *Am Zool.* (1965) 5:713.
48. Spielman DA. *Spectral Graph Theory: The Laplacian.* [Lecture Notes] (2009). Available online at: https://www.cs.yale.edu/homes/spielman/561/2009/lect02-09.pdf (accessed May 20, 2020).
49. Fisher ME. On hearing the shape of a drum. *J Combinat Theory*. (1966) 1:105–25. doi: 10.1016/S0021-9800(66)80008-X
50. Scott BA, Camacho A, Golder H, Molfino J, Kerrisk KL, Lean I, et al. The nutritive value of pasture ingested by dairy cows varies within a herd. In: *Proceedings of the 5th Australasian Dairy Science Symposium.* Camden, NSW: The University of Sydney (2014). p. 343–6.
51. Flower FC, Sanderson DJ, Weary DM. Effects of milking on dairy cow gait. *J Dairy Sci.* (2006) 89:2084–9. doi: 10.3168/jds.S0022-0302(06)72278-0
52. Beilharz RG, Mylrea PJ. Social position and movement orders of dairy heifers. *Anim Behav.* (1963) 11:529–33. doi: 10.1016/0003-3472(63)90275-6
53. Wierenga HK. Social dominance in dairy cattle and the influences of housing and management. *Appl Anim Behav Sci.* (1990) 27:201–29. doi: 10.1016/0168-1591(90)90057-K
54. Alm A, Möller J. *Welfare of Dairy Cows in AMS and Conventional Loose Housing – Differences in Behaviour and the Hormones Oxytocin and Cortisol Between Cows High or Low in Social Rank.* Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management (2004).
55. Olofsson J. *Feed availability and its effects on intake, production and behaviour in dairy cows* [Doctoral Thesis]. Swedish University of Agricultural Sciences, Uppsala, Sweden (2000).

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 14 August 2020
doi: 10.3389/fvets.2020.00480

# Relationships Between Rearing Enrichments, Range Use, and an Environmental Stressor for Free-Range Laying Hen Welfare

*Md Saiful Bari*[1,2,3], *Jeff A. Downing*[4], *Tim R. Dyall*[2], *Caroline Lee*[2] *and Dana L. M. Campbell*[2]*

[1] School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia, [2] Agriculture and Food, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Armidale, NSW, Australia, [3] Department of Dairy and Poultry Science, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh, [4] Faculty of Veterinary Science, School of Life and Environmental Science, University of Sydney, Sydney, NSW, Australia

Enrichments during pullet rearing may improve adaptation and welfare of hens as they move from indoor rearing to a free-range system. Individual variation in outdoor ranging may also affect welfare. This study assessed the effects of rearing enrichments and an imposed environmental stressor on hen welfare and egg quality along with the association of welfare with ranging. Hy-Line Brown® chicks ($n = 1,386$) were reared indoors until 16 weeks with 3 enrichment treatments including a "control" group with standard floor litter, a "novelty" group that received novel objects that were changed weekly, and a "structural" group with H-shaped perching structures. Pullets were then moved to a free-range system with three replicates of each rearing treatment. Daily ranging was individually tracked from 25 to 64 weeks via radiofrequency identification technology. Individual hen welfare assessments were performed at 25, 33, 43, 56, and 64 weeks and correlated with ranging time prior to these dates. At 44 weeks, the range area was reduced by 80% for 11 days to induce stress. Changes in ranging behavior, albumen corticosterone concentrations and egg quality were evaluated. GLMMs showed significant interactions between hen age and rearing treatment for live weight, number of comb wounds, plumage coverage, and toenail length (all $P \leq 0.003$), with the enriched hens showing more consistent live weight at the later ages, fewer comb wounds at 33 weeks, and better plumage coverage at the later ages, whereas the structural hens had shorter toenails as age increased. Plumage coverage showed a positive relationship with range use across most age points ($P < 0.0001$). Hens reduced ranging time following the imposed stressor but increased their number of visits with the lowest increase by the structural hens ($P = 0.03$). Significant interactions between rearing treatment and stressor for albumen corticosterone concentrations showed the structural hens decreased concentrations immediately post-stress, but the control and novelty groups increased ($P < 0.006$). The stressor increased or decreased values of most egg quality parameters across all rearing groups (all $P \leq 0.02$). Overall, provision of rearing enrichments and greater range use may have positive impacts on hen welfare.

**Keywords: novel objects, perching structures, range access, plumage coverage, corticosterone, RFID, behavior, egg quality**

OPEN ACCESS

***Edited by:***
*Stephanie Torrey, University of Guelph, Canada*

***Reviewed by:***
*Sabine G. Gebhardt-Henrich, University of Bern, Switzerland*
*Fernanda Machado Tahamtani, Aarhus University, Denmark*

***Correspondence:**
*Dana L. M. Campbell*
*dana.campbell@csiro.au*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *22 May 2020*
***Accepted:*** *29 June 2020*
***Published:*** *14 August 2020*

***Citation:***
*Bari MS, Downing JA, Dyall TR, Lee C and Campbell DLM (2020) Relationships Between Rearing Enrichments, Range Use, and an Environmental Stressor for Free-Range Laying Hen Welfare. Front. Vet. Sci. 7:480. doi: 10.3389/fvets.2020.00480*

## INTRODUCTION

Animal welfare concerns are prevalent within the consumer community with apprehensions regarding the housing and management of livestock and desires for improvements that result in greater well-being for production animals (1–4). Specifically, in the poultry sector, free-range egg production is increasing as consumers perceive these hens produce tastier, healthier (5, 6), and more welfare-friendly (2, 7) eggs. Consumers believe that fresh air and outdoor access for birds in the free-range system improve hen welfare (3). However, laying hens demonstrate marked individual dissimilarities in range use when provided with outdoor access, which may result in individual differences in welfare (8). Outdoor access and time spent ranging, a higher proportion of hens ranging, or distance of ranging by free-range hens may result in some welfare benefits to the birds. This may include improved plumage coverage (9–11), reduced incidences of severe feather pecking (12), reduced footpad lesions (10) and reduced toenail length (13, 14). However, Larsen et al. (15) found limited association between frequency of range access and comb color, beak, footpad, and plumage condition although hens that ranged farther from the shed did have darker combs and less beak damage. In a sample of hens from the larger flock used in the current study, high outdoor access resulted in improved plumage coverage, a reduced number of pecking comb wounds, and reduced toenail length toward the end of the production cycle (16). There was also a negative relationship between ranging and body weight (specifically fat and muscle) in hens that spent the longest time outside (16). However, other research has shown limited relationships between body weight and range use (15, 17). Thus, research specifically examining the longitudinal relationship between individual range use patterns and welfare parameters will provide further insight.

In Australia, pullets reared for free-range systems cannot go outdoors due to health risks and the sheds not being designed accordingly, whereas adults have range access. This dissimilarity between rearing and adult housing might affect their adaptation to the range and subsequent welfare as similar rearing and laying housing environments are recommended for hens (18) to achieve better health and welfare outcomes. Enrichments during pullet rearing might contribute to overcoming the constraint of indoor rearing for free-range hens. For example, providing periodically altered novel objects may increase the adaptation to unpredictable environments as could be experienced during outdoor ranging as adults (19), or placing perching structures in the pullet shed may improve spatial navigation (20). More enriched pullet housing might also reduce stress and improve adaptability (21). In a previous study carried out at the same facility as the current study, chicks were provided with a variety of enrichments for the first 3 weeks of life compared with standard floor litter (19). When environmental stressors were applied, the enriched hens showed lower albumen corticosterone responses compared with the non-enriched hens indicating a reduced stress response (19).

Environmental stressors can have negative impacts on the production and welfare of laying hens. Common stressors include high stocking density, changes in management practice, changes in the social environment, or changes in resource access and can result in physiological welfare impacts such as increased stress hormones and/or changes in behavioral patterns (19, 22–24) although not in all cases (25). The impacts of these stressors may also manifest as changes in egg quality where varying parameters have been shown to be impacted by dietary corticosterone (26) or environmental stressors such as temperature and infection (27). Other environmental causes of acute or chronic stress in laying hens may result in changes in their egg quality.

In this context, the study was performed to assess the effect of rearing enrichments on, and associations of individual ranging patterns with, welfare parameters of free-range laying hens across the flock cycle along with hens' adaptability to an environmental stressor. We predicted better welfare in high outdoor ranging birds over the indoor hens along with better welfare and adaptability of the hens enriched during rearing than the control hens.

## MATERIALS AND METHODS

### Ethical Statement

The research procedures were approved by the Animal Ethics Committee of the University of New England, Australia (AEC17-092).

### Animals

The study was conducted at the Rob Cumming Poultry Innovation Centre (indoors) and Laureldale poultry facility (free-range) of the University of New England, Armidale, NSW, Australia, using a total of 1386 Hy-Line® Brown layers. Surplus chicks were delivered in error and thus a total of 1,700 chicks were reared, but only 1,386 were transferred to the free-range facility. Surplus pullets that were of comparatively higher/lower body weight at 15 weeks of age and other randomly selected pullets from each pen were rehomed. A subsample of these hens at the end of the production cycle was reported on in Bari et al. (16) with similar data collection methods applied as described in the current study. The chicks and pullets were reared indoors within nine pens (6.2 m L × 3.2 m W) across three separate rooms up to 16 weeks of age, before being moved to the free-range facility and housed in nine pens within a single shed. The chicks and pullets were exposed to three enrichment treatments including a control group with a standard floor litter of rice hulls and no extra materials, a novelty group with different objects such as balls, bottles, bricks, brooms, brushes, buckets, containers, pet toys, and plastic pipes, that were added and changed weekly, and a structural group with four custom-designed H-shaped perching structures (L, W, H = 0.60 m) with two solid panels and one open-framed side. To visually isolate birds of each treatment group, shadecloth was hung on the wire pen dividers with each room having one replicate of each treatment ($n$ = 3 replicates/treatment). The birds were provided *ad libitum* commercial mash feed placed in manual round feeders along with *ad libitum* water access from automatic nipple drinkers. The pullet density was approximately 15 kg/m$^2$ (∼9 birds/m$^2$) (average 174–190 pullets/pen) at 16 weeks of age.

All of the resources were provided to meet the requirements of the current Australian Model Code of Practice for the Welfare of Animals–Domestic Poultry (28). The management schedules of temperature and lighting were maintained as per the recommendations of the Hy-Line® Brown alternative management guidelines (29). However, as the pullets were intended to move outside in a free-range house as adults, artificial LED lighting was maintained at 100 lux. No cooling system was available, but mechanical ventilation with heating was provided as needed. Chicks were vaccinated as per regulatory requirements and standard recommendations including vaccination against Newcastle disease, Marek's disease, fowl pox, fowl cholera, egg drop syndrome, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, infectious bronchitis, infectious larngotracheitis, and avian encephalomyelitis. The chicks were also infra-red beak-trimmed at the hatchery.

The pullets at 16 weeks of age were re-housed in the Laureldale free-range facility across 9 indoor pens ($n = 154$ hens/pen; 3.6 m W × 4.8 m L) each with outdoor access via pop-holes. The pullets from different replicates of each treatment were socially re-mixed within their rearing treatments. Shadecloth visually isolated the indoor pens and outdoor range areas from each other. Rice hulls were used as floor litter, and a complete litter replacement was done at the mid-point of the flock cycle. Each pen was provided with nest boxes (two small and one large nest box), perches, two round hanging feeders, and water nipples to meet model code guidelines.

From 16 weeks of age onwards, the artificial LED lighting gradually increased to 16 h light and 8 h darkness by 30 weeks of age with an average light intensity of 10.0 (±0.84 SE) lux (Lutron Light Meter, LX-112850; Lutron Electronic Enterprise CO., Ltd, Taipei, Taiwan) for each pen as measured at birds' eye height from three pen locations (front, middle, back) when the pop-holes were closed. This light intensity (lux) was the highest that could be achieved with the shed lighting system. There was no automatic temperature and humidity control in the shed, but it was mechanically fan-ventilated.

For outdoor ranging, each pen was connected to an outdoor area (31 m L × 3.6 m W for each pen) that was accessed by the hens via two pop-hole openings (18 cm W × 36 cm H). The range area just after the pop-holes was 1.1 m of concrete path, then 1.6 m of river rock followed by a grassed area with no additional trees or shelter. The grassed area became bare dirt following both hen access and the winter season. Hens were provided access to the outdoor area from 25 weeks of age (May 2018) for most of the daytime via automatic opening and closing of the pop-holes. The pop-holes opened at 9:15 a.m. and closed after sunset daily. This equated to ∼9 h of available ranging time daily across winter followed by ∼11 h of available ranging time daily after daylight saving time began (October 2018 until February 2019).

## Radio-Frequency Identification System

Radio-frequency identification (RFID) systems (17) were placed within the pop-holes to track the hens' movement in and out of the pop-holes. The RFID systems were designed and supported by Microchips Australia Pty Ltd (Keysborough, VIC, Australia) with equipment developed and manufactured by Dorset Identification B.V. (Aalten, the Netherlands) using Trovan® technology. All hens were banded with microchips (Trovan® Unique ID 100 (FDX-A): operating frequency 128 kHz; Microchips Australia Pty Ltd) glued into adjustable leg bands (Roxan Developments Ltd, Selkirk, Scotland) with the system recording the date and time of each tagged bird passing through and in which direction (onto the range, or into the pen) with a precision of 0.024 s (maximum detection velocity 9.3 m/s). The individual ranging data were collected daily from 25 until 64 weeks of age (excluding some days when there was a technical malfunction or when there were experimental processes such as the weighing/scoring of hens).

## Extraction of Ranging Data

The individual-hen daily RFID data throughout the laying cycle from 25 to 64 weeks of age were collated into four daily-average sets to match the periodic welfare scoring of the hens (see section Individual Welfare Assessment); averages at 33 weeks (32 days up to 12 June 2018), 43 weeks (55 days up to 20 August 2018), 56 weeks (48 days up to 20 November 2018), and 64 weeks (70 days up to 30 January 2019) of age. The data were run through a custom-designed software program written in the "Delphi" language (Bryce Little, Agriculture and Food, CSIRO, St Lucia, QLD, Australia) that filtered out any unpaired or "false" readings that may occur if, for example, a hen sits inside the pop hole but does not complete a full transition onto the range or back into the pen. The software program then summarized the mean daily time (hours) outdoors per day for each of the hens across the different age periods. To assess the effect of the implemented stressor (shrinkage of ranging area, see section Environmental Stressor) on ranging behavior (time outside and also the number of visits to the range), the individual-hen data 10 days before the stressor was applied and 10 days during the stress were also compiled (ranging data during the stressor period were not included in the summaries for the welfare scoring age points).

## Individual Welfare Assessment

The welfare assessment of all hens was done individually at five age points including 25, 33, 43, 56, and 64 weeks of age. All hens were scored inside under bright working lights by the same trained scorer who was not blind to the rearing treatments but was unaware of individual hen ranging patterns. All the hens were weighed individually using electronic hanging scales (BAT1; VEIT Electronics, Moravany, Czech Republic). The external welfare parameters of feather loss at different body parts (neck, chest, back, wing, vent, tail) and footpad lesions were assessed using the scoring system described by Tauson et al. (30). In this scoring system, four scores were available for feather coverage where a score of 4 indicated minimal feather damage, and a score of 1 indicated no plumage, just bare skin. The back of the neck was scored separately from the front of the neck which was not included in the analyses as the majority of damage on the neck front was believed to have resulted from rubbing on the feeder rims rather than pecking damage. A maximum score of 24 could be obtained for feather condition across six body parts. Footpad lesions were scored as a four for a normal footpad with no lesions or dermatitis and a score of 1 for swollen,

infected bumblefoot. The exact number of fresh or healing comb wounds was also counted (comb wounds were easily visible under the lights regardless of variation in comb color), and toenail length was measured in mm using a seamstress tape measure. Beaks were scored as 0, 1, or 2 indicating no, mild, or moderate damage, respectively. Beak damage was scored based on the evenness of the upper and lower mandibles including overgrowth or deformities which may have resulted from the day-old beak trimming procedure. The keels of each hen were scored by palpation as 1, 2, or 3 indicating normal (no damage), mild, or moderate damage, respectively. The birds were also examined for any other external signs of injury or illness such as a swollen abdomen, an enlarged crop or prolapse.

The mortality of hens was counted throughout the flock cycle. Hen mortality was recorded if a hen died, was euthanized, or rehomed if severely feather-pecked. A total of 28 hens were recorded throughout the cycle as the flock mortality of which nine were from the control group; nine, from the novelty group; and nine, from the structural group of rearing treatments.

## Environmental Stressor

The imposed environmental stressor for this trial was a reduction in available range area, similar to that applied in a previous study (19). The total outdoor area for each pen was reduced, using shadecloth, to ∼20% of its original size (from 31 to 6 m L). The range area was reduced for 11 days from 44 to 45 weeks of age with egg measurements (see following sections on Egg Quality and Albumen Corticosterone) taken before the range shrinkage, the first days of shrinkage (immediate stress), and at the end of the stressor period (prolonged stress).

## Egg Quality

A total of 810 eggs were sampled at three time points with 270 sampled per time point (30/pen), collected randomly from all of the laying locations including small nests, large nests, and floors of the pens. The dirty eggs were excluded. The samples were first collected 4 days prior to stressor implementation as baseline samples; the same number of eggs was collected on Day 3 of shrinkage (immediate stress) and Day 10 of shrinkage as the prolonged stress samples. All the egg samples were individually tested for egg quality parameters including shell reflectivity, egg weight, breaking strength by quasi-static compression, shell deformation to breaking point, albumen height, Haugh Unit, yolk color score, shell weight, and shell thickness [Egg quality equipment; Technical Services and Supplies (TSS), Dunnington, York, UK]. Yolk color was measured digitally as a score based on color intensity corresponding to the DSM YolkFan (TSS equipment). Empty eggshells were then washed and left to dry for 24 h. The thickness of dried shells was measured at the eggshell equator in three places using a custom-made gauge based on a Mitutoyo Dial Comparator gauge (Model 2109-10). All the measurements of eggs were made on the day of collection (except eggshell thickness) by personnel blinded to the rearing treatment of the birds.

## Albumen Corticosterone

For the evaluation of concentrations of albumen corticosterone, a total of 50 eggs from each of the nine pens were sampled at three stages on the same days as the egg quality measurements; Day 4 prior to range shrinkage, and Days 3 and 10 following shrinkage. Eggs were collected from all laying locations but excessively dirty eggs were excluded. On the day of collection, the eggs were opened individually, the yolk was separated out and then the albumen was weighed and stored at −20°C until assessment using the validated radioimmunoassay reported by Downing and Bryden (31). All the egg corticosterone samples were analyzed blindly to the rearing treatments and implemented stressors.

## Data and Statistical Analyses

Statistical analyses were conducted in JMP® 14.0 (SAS Institute, Cary NC, USA) with α set at 0.05. The individual hen or sampled egg was the experimental unit. Data were transformed where needed but the raw values are presented in the tables and graphs. Non-significant interactions were removed from the final models. *Post-hoc* Student's *t*-tests were applied to the least-squares means where significant differences were present.

The welfare scoring data including the live weight, number of comb wounds, beak score, keel score, plumage score (total), and toenail length at different age points (25, 33, 43, 56, 64 weeks) throughout the laying cycle for individual hens from different rearing treatments were compiled ($n$ = 6,876 data points/welfare parameter except for the beak score data which had $n$ = 5,492 data points as beaks were not scored at the 25 week assessment date). The number of comb wounds and plumage score data were square-root-transformed, and the toenail length data were $\log_{10}$-transformed. For live weight, number of comb wounds, plumage score, and toenail length, general linear mixed models were fitted, with rearing treatment, age of hen, and their interaction as fixed effects and bird ID nested within pen nested with rearing treatment and pen nested within rearing treatment as random effects. The ordinal beak, keel and footpad scores were analyzed using an ordinal logistic regression with rearing treatments, age of hen and their interaction as fixed effects. The mean daily ranging time (h) of individual birds across all rearing treatments combined were correlated with the welfare parameters of live weight, beak score, keel score, number of comb wounds, plumage score (total), and toenail length of each bird using simple linear regressions separately for each age point. Ordinal logistic regressions were applied to the beak, keel, and footpad scores and ranging data. The *r*-values for each parameter were also calculated to display the direction of the relationship (*rho* values for the ordinal data).

The egg albumen corticosterone concentration data across the stressor period were compiled for the three different rearing treatments ($n$ = 1,329 data points, 21 samples could not be accurately processed). A general linear mixed model was fitted, with rearing treatments, the stressor treatment, and their interaction as fixed effects and pen nested with rearing treatments as a random effect.

The average outdoor ranging time (h) per day and the average number of visits per day before the stress treatment and after applying stress were compiled for individual birds. The differences in ranging time (h) and number of visits per day were calculated per bird ($n$ = 1,303 data points as ranging data were unavailable for hens that stayed inside). A positive difference number indicated a decrease in the number of visits/ranging time

(h), and a negative difference number indicated an increase in the number of visits/ranging time (h) during the stressor period. General linear mixed models were fitted, with rearing treatment as a fixed effect and bird ID nested within pen nested with rearing treatment as a random effect.

Egg quality parameters measured from hens prior to stressing (baseline), immediate stress, and at prolonged stress were compiled based on the individual egg sampled ($n = 810$ data points: 30 eggs × 9 pens × 3 time collections). The values obtained for shell deformation and shell thickness were $\log_{10}$ transformed to improve normality. Percent shell reflectivity data were converted to proportions and logit transformed, and yolk color score data were square-root-transformed. General linear mixed models were fitted, with stressor time point and rearing treatment as fixed effects including their interaction, and pen nested within rearing treatments as a random effect.

# RESULTS

## Welfare Assessment

There was a significant interaction between hen age and rearing treatments for the live weight of free-range hens [$F_{(8, 5475)} = 5.46$, $P < 0.003$] where the control hens showed a smaller increase in body weight at 56 weeks of age and a greater reduction at 64 weeks of age compared to the structural and novelty hens (**Figure 1**). However, all hens showed similar trends across age with increases in live weight up until 56 weeks followed by a decrease at 64 weeks of age (**Figure 1**). There was also a significant interaction between hen age and rearing treatments for the average number of comb wounds [$F_{(8, 5490)} = 5.47$, $P < 0.0001$] with the control hens showing a greater increase in wound numbers at 33 weeks of age (**Figure 2**). Similar patterns of change were observed across age for all hens with an increase at 33 weeks followed by a decrease across the flock cycle (**Figure 2**). There was a significant interaction between hen age and rearing treatments for plumage coverage [$F_{(8, 5476)} = 86.43$, $P < 0.0001$], where both the novelty and structural hens had better plumage coverage than the control hens at the later ages (**Figure 3**). All hens did show a reduction in plumage coverage across age from 43 weeks onwards (**Figure 3**). There was a significant interaction between hen age and rearing treatments for toenail length [$F_{(8, 5482)} = 11.30$, $P < 0.0001$] where the structural hens had the shortest toenails at the later ages, and the novelty hens the longest (**Figure 4**). All hens showed similar changes across age with an initial decrease in toenail length followed by an increase at 56 and 64 weeks of age (**Figure 4**).

An ordinal logistic regression showed that both the age of hen (mean score ± SEM: 33 weeks 0.23 ± 0.01, 43 weeks 0.14 ± 0.01, 56 weeks 0.12 ± 0.01, 64 weeks 0.10 ± 0.01) ($\chi^2 = 104.07$, df = 4, $P < 0.0001$) and rearing treatment (mean score ± SEM: control 0.15 ± 0.01, novelty 0.08 ± 0.01, structural 0.12 ± 0.01) ($\chi^2 = 31.05$, df = 2, $P < 0.0001$) had a significant relationship with

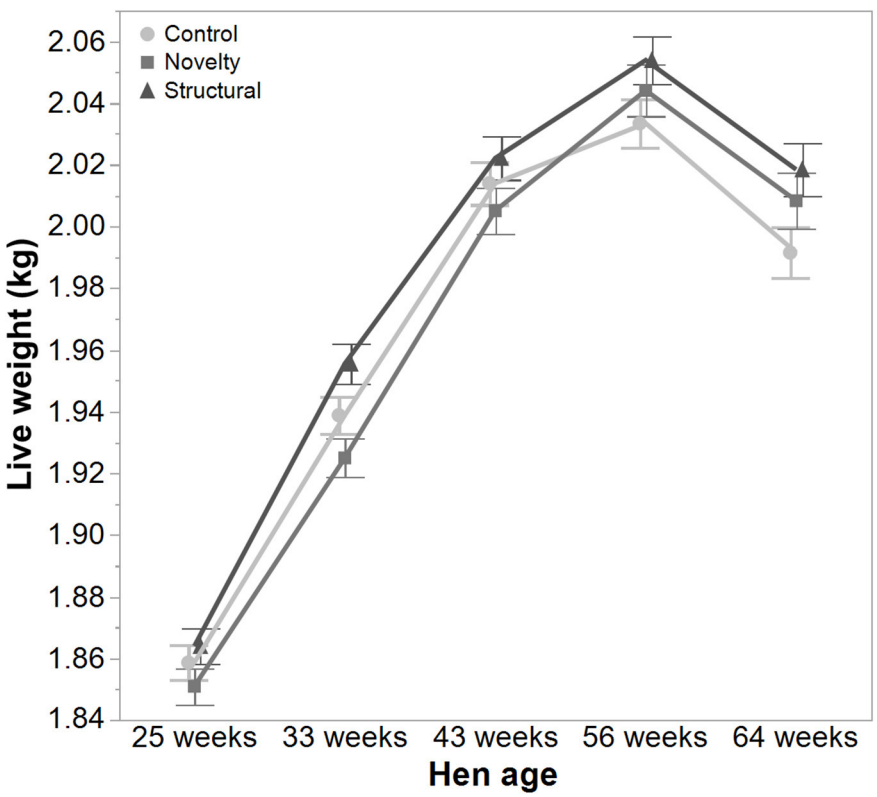


**FIGURE 1 |** The mean ± SEM of live weight (kg) of hens from different rearing treatments (control, novelty, structural) at different age points (25, 33, 43, 56, 64 weeks) in their laying cycle. The rearing treatments and age of hen interacted significantly ($P < 0.003$).

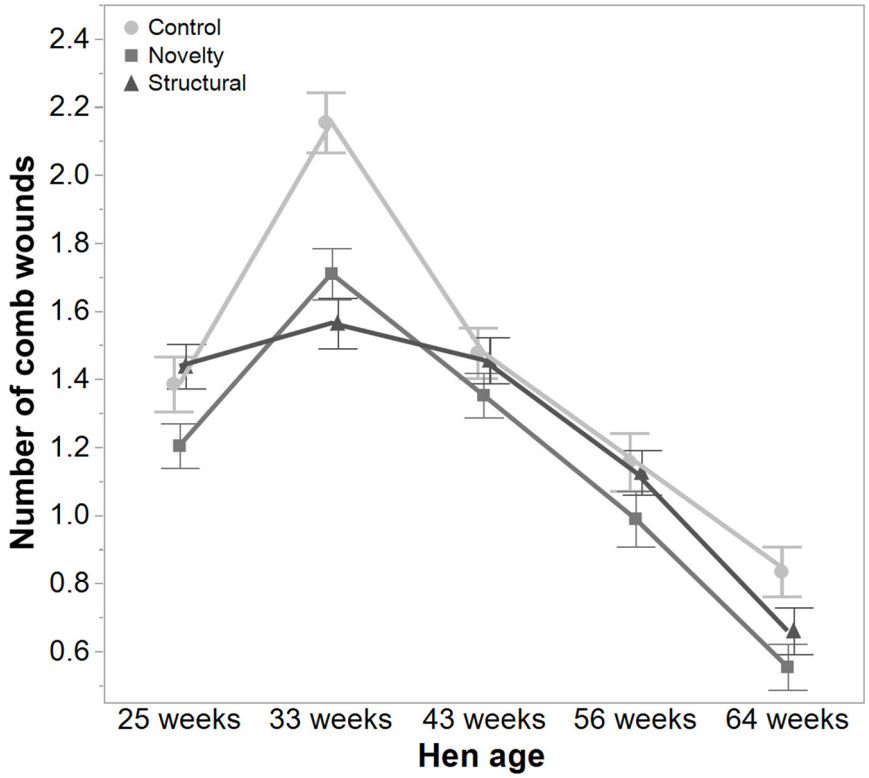


**FIGURE 2 |** The mean ± SEM of the number of comb wounds in hens from different rearing treatments (control, novelty, structural) at different age points (25, 33, 43, 56, 64 weeks) in their laying cycle. The rearing treatments and age of hen interacted significantly ($P < 0.0001$). Raw data are presented with analysis conducted on transformed data.

the beak score of free-range hens, but no significant interaction ($P = 0.10$), so this was removed from the final model. Both the age of hens (mean score ± SEM: 25 weeks: 1.03 ± 0.004, 33 weeks 1.01 ± 0.002, 43 weeks 1.05 ± 0.01, 56 weeks 1.09 ± 0.01, 64 weeks 1.09 ± 0.01) ($\chi^2 = 142.65$, df = 4, $P < 0.0001$) and rearing treatment (mean score ± SEM: control 1.05 ± 0.001, novelty 1.05 ± 0.001, structural 1.07 ± 0.001) ($\chi^2 = 13.43$, df = 2, $P = 0.001$) had a significant relationship with the keel score of free-range hens but no significant interaction ($P = 0.18$), so this was removed from the final model. The age of hens had a significant relationship ($\chi^2 = 172.92$, df = 4, $P < 0.0001$) with the footpad score (mean score ± SEM: 25 weeks: 4 ± 0, 33 weeks 3.96 ± 0.005, 43 weeks 3.98 ± 0.005, 56 weeks 3.99 ± 0.004, 64 weeks 3.93 ± 0.008), but rearing treatment did not ($\chi^2 = 4.21$, df = 4, $P = 0.12$) and there was no significant interaction ($P = 0.16$), so this was removed from the final model. Overall, across age, the beak score decreased (better beak condition), the footpad scores decreased (worse footpad condition), and the keel scores increased (worse keel condition). The structural group had higher keel scores, and the novelty group had lower beak scores than the other groups. Across the study period, few other health issues were observed, and most occurred when the hens were older. In total, the documented health issues comprised: control group: one hen observed wheezing, seven with prolapses, three with enlarged crops, and three with swollen abdomens; novelty group: six hens with prolapses; structural group: eight hens with prolapses, one with an enlarged crop, and four with swollen abdomens.

## Relationship Between Hen Welfare and Ranging

The test statistics for relationships between welfare parameters and ranging are shown in **Table 1**. There was a significant negative relationship between live weight and ranging at 56 and 64 weeks of age (both $P < 0.0001$). The beak and keel damage score of hens had significant negative and positive relationships with outdoor ranging at 43 (both $P = 0.04$) and 64 ($P = 0.0003$ and 0.0004) weeks of age, respectively. Footpad scores had significant negative relationships with outdoor ranging at 33 and 64 weeks of age (both $P \leq 0.002$), and a significant positive relationship at 43 weeks ($P = 0.03$). The number of comb wounds and ranging showed a significant negative relationship at 33 ($P = 0.0005$), 56 ($P = 0.006$) and 64 ($P < 0.0001$) weeks of age. The plumage coverage score and ranging had a significant positive relationship at 43, 56, and 64 weeks of age (all $P < 0.0001$). The toenail length of hens and ranging were significantly negatively correlated at all age points including 33, 43, 56 and 64 weeks of age (all $P < 0.0001$). Overall, ranging affected several welfare parameters but the strongest relationship ($R^2$ value) was between ranging and toenail length (**Table 1**).

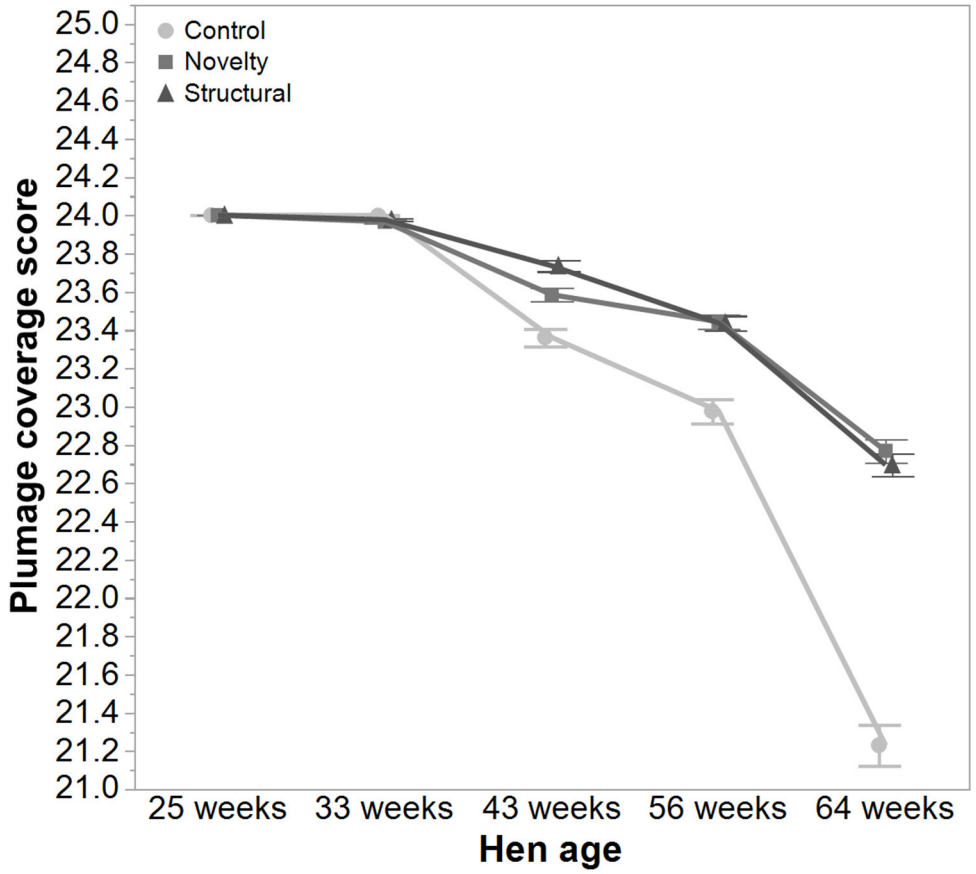


**FIGURE 3 |** The mean ± SEM of plumage score of hens from different rearing treatments (control, novelty, structural) at different age points (25, 33, 43, 56, 64 weeks) in their laying cycle. The rearing treatments and age of hen interacted significantly ($P < 0.0001$). Lower scores reflect poorer plumage condition. Raw data are presented with the analysis conducted on transformed data.

## Stressor and Rearing Treatment Effects on Ranging Behavior and Albumen Corticosterone

The average number of visits outside increased following range area shrinkage and varied between rearing treatments with a lower increase in the number of visits for the structural group hens [$F_{(2, 1300)} = 3.51$, $P = 0.03$, **Figure 5**]. In contrast, the hens' ranging time (h) decreased but did not differ significantly between the rearing treatments [$F_{(2, 1300)} = 1.41$, $P = 0.24$].

There was a significant interaction between rearing treatments and the imposed stressor treatment on the egg albumen corticosterone concentrations at 43 weeks of age [$F_{(4,1314)} = 90.29$, $P < 0.006$]. The corticosterone concentration of both the control and novelty group of hens increased immediately following the range shrinkage but then decreased at the prolonged stress time point. In contrast, the albumen corticosterone concentration in structural hens decreased immediately following the range shrinkage and then increased slightly at the prolonged stress time point (**Figure 6**).

## Stressor and Rearing Treatment Effects on Egg Quality

The rearing treatments did not significantly affect the egg quality parameters (all $P \geq 0.30$) but the stressor treatment did (**Table 2**). The eggshell reflectivity ($P < 0.0001$), egg weight (a trend at $P = 0.06$), breaking strength ($P = 0.004$), shell deformation ($P = 0.0008$), and shell weight ($P = 0.02$) all decreased as the stressor time increased. In contrast, the albumen height and Haugh unit increased across the stressor duration (both $P < 0.0001$). The yolk color score fluctuated ($P = 0.0002$) with an increase in the immediate stress period indicating darker yolks and then a decrease at the prolonged stress period corresponding to a lighter colored yolk (**Table 2**). There were no significant interactions for any of the parameters ($P \geq 0.09$) except for eggshell weight [$F_{(4,795)} = 2.63$, $P < 0.006$]. In the immediate and the prolonged stress period, the eggshell weight of the structural group showed an increase followed by a decrease in shell weight, whereas the opposite pattern was seen for the other treatment groups.

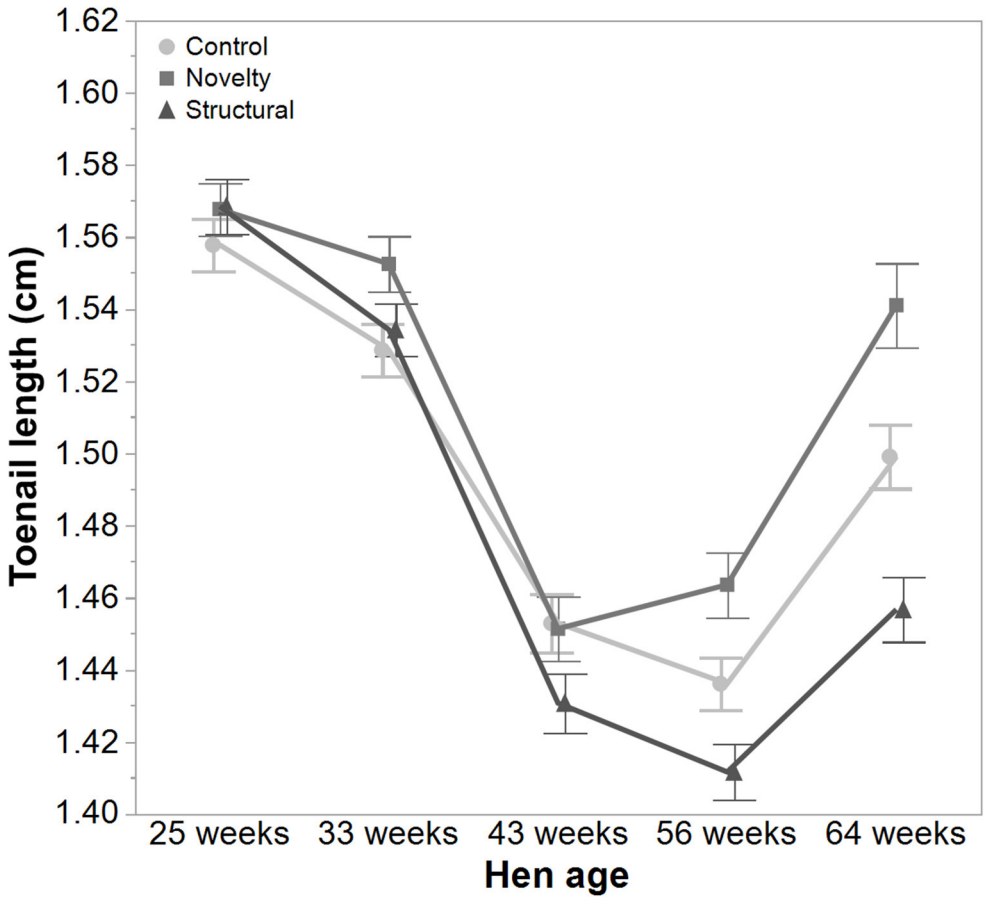


**FIGURE 4 |** The mean ± SEM of toenail length (cm) of hens from different rearing treatments (control, novelty, structural) at different age points (25, 33, 43, 56, 64 weeks) in their laying cycle. The rearing treatments and age of hen interacted significantly ($P < 0.0001$). Raw data are presented with analysis conducted on transformed data.

## DISCUSSION

We evaluated the impacts of rearing enrichments on, and associations of, outdoor ranging with welfare parameters of free-range hens across the laying cycle along with their adaptation to an imposed environmental stressor. Rearing treatments affected welfare parameters of plumage coverage, toenail length, and body weight with greater differences seen between treatments as the hens aged. Typically both types of enriched hens were different from the control hens and showed improved welfare but not exclusively across all measured parameters. The structural hens showed more keel bone damage. Results on ranging patterns from the same flock of hens showed that the structural hens spent more time outside and the novelty hens had fewer visits to the range; both enriched groups had longer individual visits than the control hens (32). Welfare parameters of body weight, comb wounds, toenail length, beak damage and footpad condition decreased with range use, and keel bone damage increased but inconsistently across the measured age points. Plumage coverage improved with range use across most age points. The average number of visits outside increased due to the imposed stressor and varied between rearing treatments with a lower increase in the number of visits in the structural group of hens. Correspondingly, the structural hens showed contrasting changes in albumen corticosterone concentrations where the corticosterone decreased immediately after the implementation of the stressor but increased in the control and novelty hens. There were clear impacts of the stressor treatment on all egg quality parameters. The limitation of only three replicates per treatment due to the confounds of the available experimental facilities must be acknowledged in the interpretation of the findings.

Enriched hens had better plumage coverage throughout the laying cycle. A subset of hens with the most extreme ranging patterns (nil, low, and high range use) from the same flock as the current study also showed better plumage in the enriched hens than the non-enriched hens at the later stage of the laying cycle (16). Plumage losses are typically the result of the feather pecking behavior of hens. Rearing enrichments might affect the development of pullets' behavior (33) such as increasing exploratory behavior (20, 34) and navigation abilities (20), subsequently affecting their movement both indoors and

**TABLE 1 |** The regression analyses of welfare parameters with outdoor ranging time (hours per day) of free-range hens at different age points across the flock cycle.

| Parameters | Hen age | *r** | $R^2$ | *F*- stats | *P* |
|---|---|---|---|---|---|
| Live weight | 33 weeks | −0.02 | 0.0006 | $F_{(1, 1381)} = 0.82$ | 0.36 |
| | 43 weeks | −0.04 | 0.002 | $F_{(1, 1373)} = 2.21$ | 0.14 |
| | 56 weeks | −0.11 | 0.01 | $F_{(1, 1374)} = 15.44$ | < 0.0001 |
| | 64 weeks | −0.11 | 0.01 | $F_{(1, 1356)} = 17.10$ | < 0.0001 |
| [a]Beak score | 33 weeks | −0.04 | 0.0009 | df = 1, $\chi^2 = 1.48$ | 0.22 |
| | 43 weeks | −0.05 | 0.004 | df = 1, $\chi^2 = 4.29$ | 0.04 |
| | 56 weeks | −0.13 | 0.02 | df = 1, $\chi^2 = 22.12$ | < 0.0001 |
| | 64 weeks | −0.10 | 0.02 | df = 1, $\chi^2 = 13.31$ | 0.0003 |
| [a]Keel score | 33 weeks | −0.002 | 0.0001 | df = 1, $\chi^2 = 0.008$ | 0.93 |
| | 43 weeks | 0.05 | 0.007 | df = 1, $\chi^2 = 4.05$ | 0.04 |
| | 56 weeks | 0.05 | 0.003 | df = 1, $\chi^2 = 2.75$ | 0.10 |
| | 64 weeks | 0.10 | 0.01 | df = 1, $\chi^2 = 12.33$ | 0.0004 |
| [a]Footpad score | 33 weeks | −0.06 | 0.02 | df = 1, $\chi^2 = 7.66$ | 0.006 |
| | 43 weeks | 0.06 | 0.02 | df = 1, $\chi^2 = 5.23$ | 0.02 |
| | 56 weeks | −0.009 | 0.004 | df = 1, $\chi^2 = 0.07$ | 0.79 |
| | 64 weeks | −0.10 | 0.02 | df = 1, $\chi^2 = 13.75$ | 0.0002 |
| Number of comb wounds | 33 weeks | −0.11 | 0.009 | $F_{(1, 1381)} = 12.17$ | 0.0005 |
| | 43 weeks | −0.007 | 0.0002 | $F_{(1, 1373)} = 0.23$ | 0.64 |
| | 56 weeks | −0.08 | 0.005 | $F_{(1, 1374)} = 7.57$ | 0.006 |
| | 64 weeks | −0.08 | 0.005 | $F_{(1, 1356)} = 6.62$ | 0.01 |
| Plumage score | 33 weeks | 0.04 | 0.002 | $F_{(1, 1381)} = 2.14$ | 0.14 |
| | 43 weeks | 0.17 | 0.03 | $F_{(1, 1373)} = 42.14$ | < 0.0001 |
| | 56 weeks | 0.25 | 0.06 | $F_{(1, 1374)} = 91.74$ | < 0.0001 |
| | 64 weeks | 0.22 | 0.05 | $F_{(1, 1356)} = 71.46$ | < 0.0001 |
| Toenail length | 33 weeks | −0.18 | 0.03 | $F_{(1, 1381)} = 43.57$ | < 0.0001 |
| | 43 weeks | −0.39 | 0.15 | $F_{(1, 1373)} = 237.92$ | < 0.0001 |
| | 56 weeks | −0.43 | 0.18 | $F_{(1, 1374)} = 304.21$ | < 0.0001 |
| | 64 weeks | −0.49 | 0.23 | $F_{(1, 1356)} = 407.50$ | < 0.0001 |

[a]*Subjected to ordinal logistic regression and Spearman's correlation.*

[*]*A correlation coefficient is included to display the direction of the relationship.*

outdoors (19). Hens that spend more time exploring and foraging outside may consequently reduce the time spent feather pecking conspecifics or be better able to avoid being pecked. As the hens from different rearing treatments also showed differences in ranging behavior (32), it is unclear whether the effects of the rearing treatments were related to behavioral differences that developed during the rearing period, if they were a consequence of the variation in range use, or a combination of both. Reductions in feather pecking behavior and/or improvements in plumage have previously been demonstrated to be associated with greater use of the range area (9, 10, 35), although Larsen et al. (15) found no association between plumage condition and individual outdoor ranging. Feather pecking might also be associated with negative affective states such as fear (36) which could be mitigated by increased exercise, a hypothesis that warrants further investigation in ranging hens. Differences in pecking behavior may be related to differences in social interactions. Early feather pecking behavior is evidenced to be associated with social exploration (37), and in a previous study with free-range hens, there were differences in synchronized group-level ranging patterns between enriched/non-enriched hens (38). In support of this, the most comb wounds were seen at 33 weeks of age and more so in the control hens than both enriched groups. This might be a result of the social restructuring in the group when they started to use the range. Pop-holes were first opened at 25 weeks of age, but range use was initially low (32). At 33 weeks of age when range use was increasing, the indoor stocking density lowered and potentially resulted in the reorganization of social hierarchies. The control hens with the most comb wounds may have been poorer at managing their social interactions.

Other welfare parameters were also associated with range use with more differences seen as the hens aged. Similarly, hours spent outside increased as the hens aged followed by a drop from 56 to 64 weeks of age which was likely affected by the summer season (32). However, although there were significant relationships for keel damage, beak condition, footpad condition, comb wound count, and body weight, the $R^2$ values were very low indicating that the ranging patterns only accounted for some of the variability that was seen. Keel bone damage was higher in the structural hens, but they also showed more ranging (32), which may have contributed to this difference

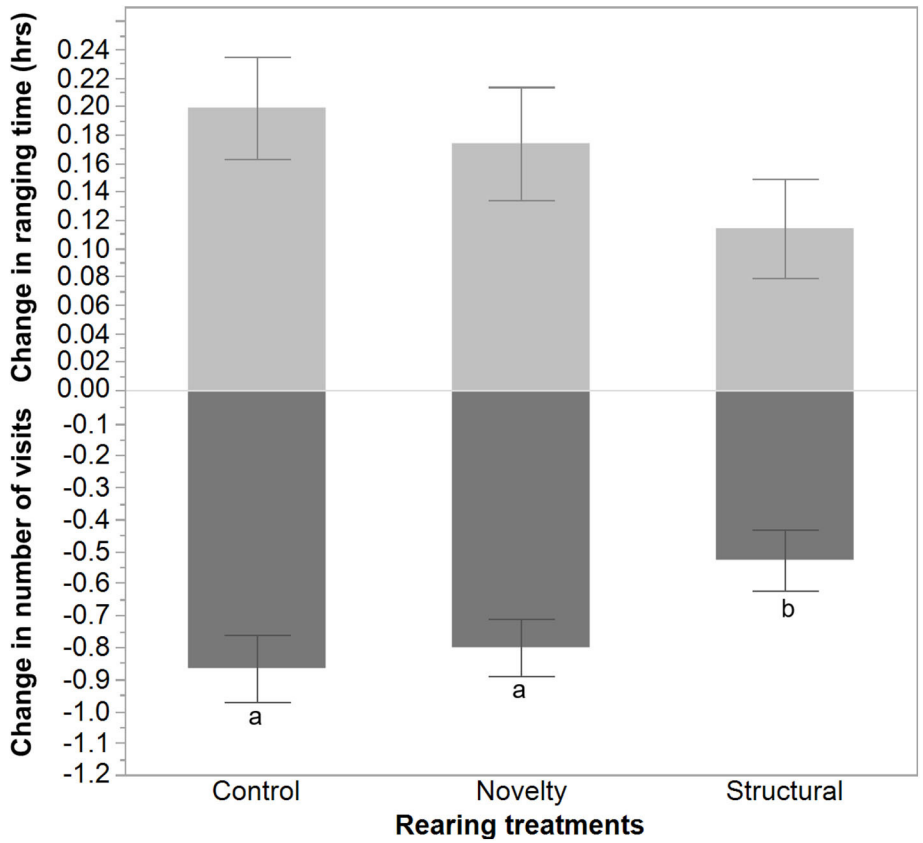


**FIGURE 5 |** The mean ± SEM of the changes in ranging time (hours) and number of visits due to the applied stress on free-range hens from different rearing treatments (control, novelty, structural). [a,b]Dissimilar superscript letters indicate significant differences between the change in the number of visits across different rearing treatments ($P < 0.05$).

between the rearing treatments. Larsen et al. (15) also found few associations between welfare indicators and range use variability in commercial free-range hens. These authors hypothesized that the choice provided in the free-range environment allows each hen to range to the degree that meets their own needs; thus, natural individual variation in ranging may not have detectable implications for welfare. The impacts of range use may, however, be stronger for hens that show more extreme ranging patterns as were selected in a subsample of hens from the current study (16). Additionally, forced ranging patterns disparate from natural choices (i.e., reducing range access hours, or forcing hens outside) may have greater effects on individual welfare, but this hypothesis remains to be tested. The clearest relationship between ranging and welfare scoring was the reduction of toenail length for hens that spent more time outside. This result has previously been demonstrated (13, 17), and it is expected that more time walking/scratching in the dirt would maintain suitable toenail lengths which can reduce the risks of getting toenails caught in the structure of the system.

The applied environmental stressor impacted the hens' ranging by decreasing the ranging time outdoors while increasing the number of visits outside. There was no effect of rearing treatment on the change in time spent outside, but the structural hens showed a lower increase in the number of outdoor visits. These ranging behavior results are similar to those of a previous study conducted in the same facility that applied the same stressor to smaller flocks of hens exposed to enrichments (or not) for the first 3 weeks of life (19). However, in this previous study, the enriched hens (visual, auditory, structural enrichments) showed a greater increase in the number of visits relative to non-enriched hens (19). Physiologically, the structural hens actually showed a decrease in albumen corticosterone when sampled 3 days after the range area was reduced, compared with an increase in the control and novelty hens. This result is similar to the corticosterone responses following the first week of range access in the same flock of hens where the structural hens showed the smallest increase compared with the other treatment groups and had higher baseline levels (32). In the previous study the enriched hens also showed lower increases in corticosterone following imposed stressors (19). The structural hens in the current study may have been more adaptable to environmental change showing comparatively lower behavioral modification and a lower physiological stress response; however, the mechanism for this is unclear. The lower physiological response and comparatively lower behavioral response does not align with coping styles, as in both active and passive

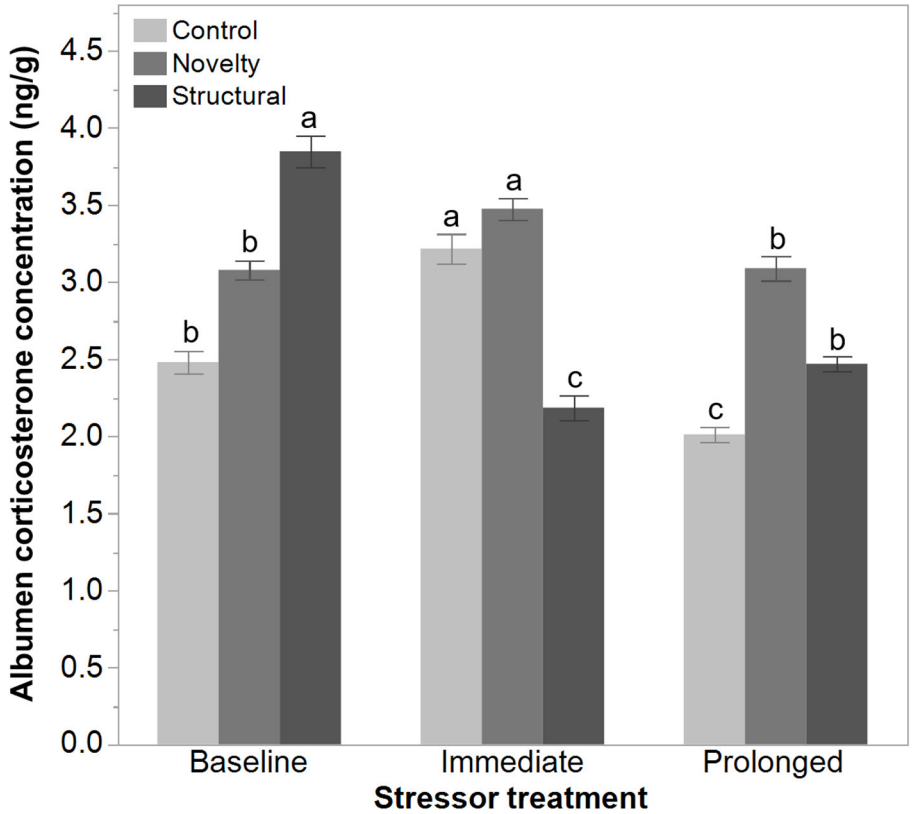


**FIGURE 6 |** The albumen corticosterone concentrations (ng/g) of eggs from 43 to 45-week old free-range hens from different rearing treatments (control, novelty, structural) across an implemented stressor period (baseline, immediate, prolonged). The interaction between rearing and stressor treatments was significant ($P < 0.006$) but $^{a,b,c}$dissimilar superscript letters indicate the significant difference across stressor time only.

**TABLE 2 |** The least squares means (LSM) ± standard error of the mean (SEM) of egg quality parameters across the implemented stressor period (baseline, immediate, prolonged).

| Parameters | Stress treatment | | | SEM | *F*, df | *P* |
|---|---|---|---|---|---|---|
| | Baseline | Immediate | Prolonged | | | |
| Shell reflectivity | $30.75^{a}$ | $26.30^{b}$ | $26.71^{b}$ | 0.25 | $F_{(2, 799)} = 96.08$ | < 0.0001 |
| Egg weight (g) | $62.69^{a}$ | $61.87^{b}$ | $61.97^{ab}$ | 0.35 | $F_{(2, 799)} = 2.86$ | 0.06 |
| Albumen height (mm) | $9.96^{b}$ | $10.36^{a}$ | $10.55^{a}$ | 0.13 | $F_{(2, 799)} = 13.61$ | < 0.0001 |
| Haugh unit | $98.10^{b}$ | $100.30^{a}$ | $101.00^{a}$ | 0.60 | $F_{(2, 799)} = 16.05$ | < 0.0001 |
| Yolk color score | $10.34^{c}$ | $10.65^{a}$ | $10.50^{b}$ | 0.09 | $F_{(2, 799)} = 8.52$ | 0.0002 |
| Breaking strength (N) | $46.85^{a}$ | $45.10^{b}$ | $45.06^{b}$ | 0.53 | $F_{(2, 799)} = 5.48$ | 0.004 |
| Shell deformation (mm) | $0.29^{a}$ | $0.28^{b}$ | $0.27^{b}$ | 0.004 | $F_{(2, 799)} = 7.23$ | 0.0008 |
| Shell weight (g) | $6.13^{a}$ | $6.03^{b}$ | $6.04^{b}$ | 0.03 | $F_{(2, 795)} = 3.99$ | 0.02 |
| Shell thickness (mm) | 0.43 | 0.43 | 0.43 | 0.002 | $F_{(2, 799)} = 0.61$ | 0.54 |

$^{a-c}$*Means with different superscript letters in each row differ significantly ($P < 0.05$). Raw data are presented in the table with some analyses conducted on transformed data.*

responses, the direction of change between behavioral and physiological parameters oppose each other (39). The structural hens may have developed improved adaptability through the perching structures during rearing that included both height and opaque panels. This may have enabled the pullets to exhibit avoidance behaviors as needed (e.g., perching as a predator avoidance strategy) which stimulated coping. Further research would be needed to explore this idea. The higher degree of outdoor ranging prior to implementation of the stressor may have also meant these hens were getting more exercise, which modified the functioning of their hypothalamic pituitary adrenal axis and advanced their rate of physiological adaptation to

the stressor (40). The complex relationship between baseline metabolism and glucocorticoids may have been impacted by typical ranging differences between the treatment groups (41, 42). All hens reduced ranging time, but the control and novelty hens increased their visits, whereas the structural hens did not to the same degree; thus, their overall ranging activity was lower during the stressor period. The validated radioimmunoassay to determine the corticosterone concentrations used antiserum that does have some cross-reactivities to other steroids (31), so it is unclear to what degree these may have affected the results. Blood profiles in future testing could be more informative or provide additional measures (41) but require invasive sampling techniques. Further studies could also measure the rate of adaptation following the removal of an imposed stressor, which was not assessed in this study, including measuring the use of the available range area rather than just time and visits outside.

It is possible that the structural enrichments during rearing resulted in neurological changes such as greater hemispheric flexibility that improved adaptive responses to their environments (43). Previous comparisons between cage-reared and aviary-reared hens showed functional lateralization in the hippocampus and caudolateral nidopallium but no differences between the rearing treatments, although all birds were in similar environments for the first 4 weeks of rearing (44). Campbell et al. (45) found no differences in the telencephalon or hippocampal volume between enriched-reared and non-enriched-reared hens. However, multiple studies in rodents have demonstrated increased synaptic plasticity in the hippocampus of animals exposed to enrichments, particularly short-term (46). The impacts of rearing on neural maturation warrant further investigation.

Environmental stressors did affect egg quality with eggshell reflectivity, egg weight, breaking strength, shell deformation, and shell weight showing decreases, but albumen height and Haugh units showed increases across the stressor period. However, distinct from the rearing treatment effects in behavioral and corticosterone change, the effects on egg quality were similar across all groups of hens. Some of these effects of stress on egg parameters were similar to impacts of heat stress and disease on egg quality (27, 47, 48). Decreases in yolk color also correspond with the effects of dietary corticosterone but the increased albumen height is opposite to previous reports of corticosterone supplementation (26) or heat stress (49). The implemented stressor and changes in corticosterone may have affected albumen proteins (50), but changes in activity levels (ranging behavior) and potentially feed intake may have also had impacts on nutrient allocation as rearing treatment differentially affected corticosterone concentrations but not egg quality parameters. The egg weight and shell characteristics including eggshell color were decreased due to stress, which might be related to reduced feed intake, particularly calcium which could have affected breaking strength, shell deformation, and shell weight. However, feed intake was not measured in this study, and thus, further research is warranted to clarify this.

## CONCLUSION

Overall, enrichments in rearing provided welfare benefits at some age points, including better plumage coverage, fewer comb wounds, and shorter toenails, but this was likely associated with the differences in ranging also seen between the rearing treatment groups. Ranging was related to primarily improved welfare parameters of free-range hens, but these relationships had high individual variability. Structural enrichments may have improved adaptation by minimizing both behavioral changes and immediate physiological stress responses. Change in resource access decreased egg quality, but rearing enrichments did not minimize these effects. Rearing enrichments along with optimum range access could be recommended for positive effects on hen welfare. However, this study only had three replicates per treatment due to the limitations of the experimental facilities, and thus, longitudinal studies with increased replicates and in commercial settings to clarify the relationship between individual range use and welfare parameters are warranted.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of New England Animal Ethics Committee.

## AUTHOR CONTRIBUTIONS

DC conceived and designed the experiment. MB and DC collected and analyzed the data, and prepared the figures and tables. MB drafted the manuscript. JD performed the corticosterone analyses. TD and CL assisted in data collection, experimentation, and project administration. MB and DC revised the manuscript, all authors approved the final version, and contributed significantly to this manuscript.

## FUNDING

Funding was provided by Poultry Hub Australia (Grant No: 2017-20). The University of New England (UNE) and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) supported MB with postgraduate scholarships.

## ACKNOWLEDGMENTS

We are grateful for the technical expertise of B. Dawson and A. Gasbarri at the Science & Engineering Workshop, UNE. We would also like to thank A. Cohen-Barnhouse (UNE) and the post-graduate students and staff of UNE and CSIRO for their contribution in the husbandry, technical support, and data collection for the experiment. Thank you to the people that rehomed the birds during and at the conclusion of the trial.

## REFERENCES


1. Edge M, Barnett J. Development of animal welfare standards for the livestock transport industry: process, challenges, and implementation. *J Vet Behav Clin Appl Res.* (2009) 4:187–92. doi: 10.1016/j.jveb.2009.07.001
2. Vanhonacker F, Van Poucke E, Tuyttens F, Verbeke W. Citizens' views on farm animal welfare and related information provision: exploratory insights from Flanders, Belgium. *J Agric Environ Ethics.* (2010) 23:551–69. doi: 10.1007/s10806-010-9235-9
3. Pettersson IC, Weeks CA, Wilson LRM, Nicol CJ. Consumer perceptions of free-range laying hen welfare. *Br Food J.* (2016) 118:1999–2013. doi: 10.1108/BFJ-02-2016-0065
4. Moffat K, Murphy S, Boughen N. *Australian Egg Industry Community Research Report 2019*. CSIRO (2020).
5. Heng Y, Peterson HH, Li X. Consumer attitudes toward farm-animal welfare: the case of laying hens. *J Agr Resour Econ.* (2013) 38:418–34. doi: 10.22004/ag.econ.165936
6. Bray HJ, Ankeny RA. Happy chickens lay tastier eggs: motivations for buying free-range eggs in Australia. *Anthrozoös.* (2017) 30:213–26. doi: 10.1080/08927936.2017.1310986
7. Bennett RM, Jones PJ, Nicol CJ, Tranter RB, Weeks CA. Consumer attitudes to injurious pecking in free range egg production. *Anim Welf.* (2016) 25:91–100. doi: 10.7120/09627286.25.1.091
8. Campbell DLM, Bari MS, Rault J-L. Free-range egg production: its implications for hen welfare. *Anim Prod Sci.* (2020). doi: 10.1071/AN19576. [Epub ahead of print].
9. Chielo LI, Pike T, Cooper J. Ranging behaviour of commercial free-range laying hens. *Animals.* (2016) 6:1–13. doi: 10.3390/ani6050028
10. Rodriguez-Aurrekoetxea A, Estevez I. Use of space and its impact on the welfare of laying hens in a commercial free-range system. *Poult Sci.* (2016) 95:2503–13. doi: 10.3382/ps/pew238
11. Pettersson IC, Weeks CA, Nicol CJ. Provision of a resource package reduces feather pecking and improves ranging distribution on free-range layer farms. *Appl Anim Behav Sci.* (2017) 195:60–66. doi: 10.1016/j.applanim.2017.06.007
12. Lambton SL, Knowles TG, Yorke C, Nicol CJ. The risk factors affecting the development of gentle and severe feather pecking in loose housed laying hens. *Appl Anim Behav Sci.* (2010) 123:32–42. doi: 10.1016/j.applanim.2009.12.010
13. Yilmaz Dikmen B, Ipek A, Sahan Ü, Petek M, Sözcü A. Egg production and welfare of laying hens kept in different housing systems (conventional, enriched cage, and free range). *Poult Sci.* (2016) 95:1564–72. doi: 10.3382/ps/pew082
14. Campbell DLM, Hinch GN, Downing JA, Lee C. Outdoor stocking density in free-range laying hens: effects on behaviour and welfare. *Animal.* (2017) 11:1036–45. doi: 10.1017/S1751731116002342
15. Larsen H, Hemsworth PH, Cronin GM, Gebhardt-Henrich SG, Smith CL, Rault J-L. Relationship between welfare and individual ranging behavior in commercial free-range laying hens. *Animal.* (2018) 12:2356–64. doi: 10.1017/S1751731118000022
16. Bari MS, Laurenson YCSM, Cohen-Barnhouse AM, Walkden-Brown SW, Campbell DLM. Effects of outdoor ranging on external and internal health parameters for hens from different rearing enrichments. *PeerJ.* (2020) 8:e8720. doi: 10.7717/peerj.8720
17. Campbell DLM, Hinch GN, Dyall TR, Warin L, Little BA, Lee C. Outdoor stocking density in free-range laying hens: radio-frequency identification of impacts on range use. *Animal.* (2017) 11:121–30. doi: 10.1017/S1751731116001154
18. Janczak AM, Riber AB. Review of rearing-related factors affecting the welfare of laying hens. *Poult Sci.* (2015) 94:1454–69. doi: 10.3382/ps/pev123
19. Campbell DLM, Hinch GN, Downing JA, Lee C. Early enrichment in free-range laying hens: effects on ranging behaviour, welfare and response to stressors. *Animal.* (2018) 12:575–84. doi: 10.1017/S1751731117001859
20. Gunnarsson S, Yngvesson J, Keeling LJ, Forkman B. Rearing without early access to perches impairs the spatial skills of laying hens. *Appl Anim Behav Sci.* (2000) 67:217–28. doi: 10.1016/S0168-1591(99)00125-2
21. Moe RO, Guemene D, Bakken M, Larsen HJS, Shini S, Lervik S, et al. Effects of housing conditions during the rearing and laying period on adrenal reactivity, immune response and heterophil to lymphocyte (H/L) ratios in laying hens. *Animal.* (2010) 4:1709–15. doi: 10.1017/S175173111000100X
22. Vestergaard KS, Skadhauge E, Lawson L. The stress of not being able to perform dustbathing in laying hens. *Physiol Behav.* (1997) 62:413–19. doi: 10.1016/S0031-9384(97)00041-3
23. Mirfendereski E, Jahanian R. Effects of dietary organic chromium and vitamin C supplementation on performance, immune responses, blood metabolites, and stress status of laying hens subjected to high stocking density. *Poult Sci.* (2015) 94:281–88. doi: 10.3382/ps/peu074
24. Carvalho RR, Palme R, da Silva Vasconcellos A. An integrated analysis of social stress in laying hens: the interaction between physiology, behaviour, and hierarchy. *Behav Process.* (2018) 149:43–51. doi: 10.1016/j.beproc.2018.01.016
25. Engel J, Widowski T, Tilbrook A, Butler K, Hemsworth P. The effects of floor space and nest box access on the physiology and behavior of caged laying hens. *Poult Sci.* (2019) 98:533–47. doi: 10.3382/ps/pey378
26. Kim Y-H, Kim J, Yoon H-S, Choi Y-H. Effects of dietary corticosterone on yolk colors and eggshell quality in laying hens. *Asian-Australas J Anim Sci.* (2015) 28:840–46. doi: 10.5713/ajas.14.0849
27. Mertens K, Vaesen I, Loffel J, Kemps B, Kamers B, Perianu C, et al. The transmission color value: a novel egg quality measure for recording shell color used for monitoring the stress and health status of a brown layer flock. *Poult Sci.* (2010) 89:609–17. doi: 10.3382/ps.2009-00261
28. Primary Industries Standing Committee. *Model Code of Practice for the Welfare of Animals: Domestic Poultry*. Collingwood, VIC: CSIRO Publishing (2002).
29. Hy-line. *Management Guide for Hy-Line Brown Laying Hen in Alternative System*. (2016). Available online at: https://www.hyline.com/userdocs/pages/B_ALT_COM_ENG.pdf (accessed October 23, 2017)
30. Tauson R, Kjaer J, Maria GA, Cepero R, Holm KE. Applied scoring of integument and health in laying hens. *Anim Sci Paper Rep.* (2005) 23:153–59.
31. Downing J, Bryden W. Determination of corticosterone concentrations in egg albumen: a non-invasive indicator of stress in laying hens. *Physiol Behav.* (2008) 95:381–87. doi: 10.1016/j.physbeh.2008.07.001
32. Campbell DLM, Dyall TR, Downing JA, Cohen-Barnhouse AM, Lee C. Rearing enrichments affected ranging behavior in free-range laying hens. *Front Vet Sci.* (2020) 7:446.
33. Campbell DLM, De Haas EN, Lee C. A review of environmental enrichment for laying hens during rearing in relation to their behavioral and physiological development. *Poult Sci.* (2019) 98:9–28. doi: 10.3382/ps/pey319
34. Krause ET, Naguib M, Trillmich F, Schrader L. The effects of short term enrichment on learning in chickens from a laying strain (*Gallus gallus domesticus*). *Appl Anim Behav Sci.* (2006) 101:318–27. doi: 10.1016/j.applanim.2006.02.005
35. De Koning C, Kitessa SM, Barekatain R, Drake K. Determination of range enrichment for improved hen welfare on commercial fixed-range free-range layer farms. *Anim Prod Sci.* (2018) 59:1336–48. doi: 10.1071/AN17757
36. Rodenburg TB, Van Krimpen MM, De Jong IC, De Haas EN, Kops MS, Riedstra BJ, et al. The prevention and control of feather pecking in laying hens: identifying the underlying principles. *Worlds Poult Sci J.* (2013) 69:361–74. doi: 10.1017/S0043933913000354
37. Riedstra B, Groothuis TG. Early feather pecking as a form of social exploration: the effect of group stability on feather pecking and tonic immobility in domestic chicks. *Appl Anim Behav Sci.* (2002) 77:127–38. doi: 10.1016/S0168-1591(02)00031-X
38. Campbell DLM, Horton BJ, Hinch GN. Using radio-frequency identification technology to measure synchronized ranging of free-range laying hens. *Animals.* (2018) 8:210. doi: 10.3390/ani8110210
39. Cockrem JF. Stress, corticosterone responses and avian personalities. *J Ornithol.* (2007) 148:169–78. doi: 10.1007/s10336-007-0175-8
40. Hare BD, Beierle JA, Toufexis DJ, Hammack SE, Falls WA. Exercise-associated changes in the corticosterone response to acute restraint stress: evidence for increased adrenal sensitivity and reduced corticosterone response duration. *Neuropsychopharmacology.* (2014) 39:1262–69. doi: 10.1038/npp.2013.329
41. Scanes CG. Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio. *Poult Sci.* (2015) 95:2208–15. doi: 10.3382/ps/pew137
42. McEwen BS, Wingfield JC. The concept of allostasis in biology and biomedicine. *Horm Behav.* (2003) 43:2–15. doi: 10.1016/S0018-506X(02)00024-7

43. Rogers LJ, Kaplan G. Does functional lateralization in birds have any implications for their welfare? *Symmetry.* (2019) 11:1043. doi: 10.3390/sym11081043
44. Tahamtani FM, Nordgreen J, Brantsæter M, Østby GC, Nordquist RE, Janczak AM. Does early environmental complexity influence tyrosine hydroxylase in the chicken hippocampus and "prefrontal" caudolateral nidopallium? *Front Vet Sci.* (2016) 3:8. doi: 10.3389/fvets.2016.00008
45. Campbell DLM, Talk AC, Loh ZA, Dyall TR, Lee C. Spatial cognition and range use in free-range laying hens. *Animals.* (2018) 8:26. doi: 10.3390/ani8020026
46. Ohline SM, Abraham WC. Environmental enrichment effects on synaptic and cellular physiology of hippocampal neurons. *Neuropharmacology.* (2019) 145:3–12. doi: 10.1016/j.neuropharm.2018.04.007
47. Lin H, Mertens K, Kemps B, Govaerts T, de Ketelaere B, de Baerdemaeker J, et al. New approach of testing the effect of heat stress on eggshell quality: mechanical and material properties of eggshell and membrane. *Br Poult Sci.* (2004) 45:476–82. doi: 10.1080/00071660400001173
48. Mashaly MM, Hendricks GL, Kalama MA, Gehad AE, Abbas AO, Patterson PH. Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poult Sci.* (2004) 83:889–94. doi: 10.1093/ps/83.6.889
49. Seven PT. The effects of dietary Turkish propolis and vitamin C on performance, digestibility, egg production and egg quality in laying hens under different environmental temperatures. *Asian-Australas J Ani. Sci.* (2008) 21:1164–70. doi: 10.5713/ajas.2008.70605
50. Kim J, Choi YH. Differential abundance of egg white proteins in laying hens treated with corticosterone. *J Agr Food Chem.* (2014) 62:12346–59. doi: 10.1021/jf504469t


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 14 August 2020
doi: 10.3389/fvets.2020.00446

# Rearing Enrichments Affected Ranging Behavior in Free-Range Laying Hens

*Dana L. M. Campbell*[1]*, *Tim R. Dyall*[1], *Jeff A. Downing*[2], *Andrew M. Cohen-Barnhouse*[1,3] and *Caroline Lee*[1]

[1] Agriculture and Food, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Armidale, NSW, Australia,
[2] School of Life and Environmental Science, Faculty of Veterinary Science, University of Sydney, Sydney, NSW, Australia,
[3] School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia

Within Australia, free-range systems are prevalent, but pullets destined for range access are reared indoors. This mismatch between rearing and layer housing may hinder adaptation to the free-range environment. Rearing enrichments could enhance pullet development. A total of 1,386 Hy-Line Brown® chicks were reared inside an experimental facility across 16 weeks with 3 enrichment treatments including (1) a control group with standard floor-housing, (2) a novelty group providing novel objects that changed weekly ("novelty" hens), and (3) a structural group with custom-designed H-shaped structures including opaque sides ("structural" hens). At 16 weeks of age, all pullets were leg-banded with microchips and moved to an experimental free-range system with 9 identical pens ($n = 3$/rearing treatment). From 25 to 64 weeks, individual hen daily ranging behavior was tracked via radio-frequency identification technology and grouped into 6 age periods per rearing treatment. Video footage was used to count the number of hens at different distances on the range for the first 14 days of access, and eggs were assessed for albumen corticosterone concentrations 4 days prior to ($n = 450$) and 1 week after first range access ($n = 450$). Across most age periods, the structural hens spent the most time ranging ($P \leq 0.01$), the novelty hens showed the fewest number of visits to the range ($P < 0.0001$), and both enriched hen groups had the longest maximum visit durations ($P \leq 0.02$). Range use increased with age across all treatments with only 3% of hens never going outside. All hens were initially slow to use the range area with fewer novelty hens venturing farther onto the range ($P \leq 0.03$). The structural hens had higher albumen corticosterone concentrations and variance (both $P \leq 0.004$) prior to range access. All hens showed an increase in albumen corticosterone following the first week of range access resulting in no differences between rearing treatments in means ($P = 0.92$) and variance ($P = 0.63$). Different enrichments have differing impacts on ranging behavior, but further research is needed to understand the mechanisms of effects, with differences in brain lateralization a potential hypothesis to be tested.

**Keywords: corticosterone, welfare, individual, chicken, hen, RFID, adaptability, stress**

OPEN ACCESS

***Edited by:***
*Sabine G. Gebhardt-Henrich, University of Bern, Switzerland*

***Reviewed by:***
*T. Bas Rodenburg, Wageningen University and Research, Netherlands*
*Janicke Nordgreen, Norwegian University of Life Sciences, Norway*

***Correspondence:**
*Dana L. M. Campbell*
*dana.campbell@csiro.au*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *26 February 2020*
***Accepted:*** *19 June 2020*
***Published:*** *14 August 2020*

***Citation:***
*Campbell DLM, Dyall TR, Downing JA, Cohen-Barnhouse AM and Lee C (2020) Rearing Enrichments Affected Ranging Behavior in Free-Range Laying Hens. Front. Vet. Sci. 7:446. doi: 10.3389/fvets.2020.00446*

## INTRODUCTION

In many countries, the laying hen industry is making a transition away from conventional caged housing toward alternative systems that provide hens with more resources and space to accommodate their behavioral needs. Within Australia, free-range systems are increasingly prevalent as consumers believe these systems provide better hen welfare (1), and eggs are healthier and tastier (2). However, free-range systems provide hens a choice to range or remain indoors, and in some instances, the use of the outdoor range can be low (3). This potentially limits the benefits of this system and/or could reduce consumer satisfaction. There is some evidence that higher use of the range area will improve plumage condition and footpad condition, and reduce toenail length (4, 5). Hens exhibit some important behaviors such as foraging at higher frequencies outdoors than indoors (4, 6).

There are multiple factors affecting hens' use of the range area as adults, both in terms of accessing the range and distribution in the range area (7). These include, for example, the ambient weather (8, 9), shelter on the range (10), additional enrichments on the range (11), and hen age (8, 9, 12). The range area is also a new environment that may require hens to be more adaptable compared with strictly indoor housing systems. Hens that go outside are exposed to weather variation, sunlight, predators, and large temperature fluctuations; typically, the food and water resources necessary for maintaining body condition as well as a high rate of production are located inside. Outdoor access during rearing is also a factor that affects range use as adults (13) but not in all cases (14). For hens that are not reared with outdoor access, there is often a long period (weeks) for hens to become accustomed to the range area following first pop-hole opening (12). It may be stressful to enter the outdoor environment following 16 plus weeks of being inside (first pop-hole opening age varies between commercial producers). Some hens even choose to never exit to the range, and these hens have been identified as more fearful than frequent range users (15–17).

Rearing environments for pullets are important for optimal development, adaptability, and performance as adult hens (18, 19), with studies showing that hens will better adapt to the layer system if they are reared in a similar manner. For example, hens reared in cages will better adapt to a caged layer system following transfer than hens reared in aviaries and placed into cages (20). Producers in Europe that rear free-range pullets with outdoor access report that the management of their rearing flocks to optimize adult performance is a less prominent issue than reported by producers that do not rear outdoors (21). In Australia, pullets destined for free-range systems are typically reared inside due to vaccination schedules and health risks associated with outdoor access, and the logistics of current shed designs, which do not have outdoor ranges. Thus, pullets entering free-range systems may be at a disadvantage, which could impact their range use, health, and welfare as adults. In the absence of feasible outdoor access options, enrichments in the rearing sheds could better prepare pullets for free-range housing. Enrichments can be defined as any addition to the environment that has positive impacts on behavior and/or biology of the animals (22). These can have multiple impacts on the pullets' behavioral, physical, and neurobehavioral development (23). One previous free-range chick enrichment study showed that variable physical and sensory enrichments provided for the first 3 weeks of development improved the hens' adaptation to implemented environmental stressors as adults (12), increased their degree of social flock cohesion (24), but slightly reduced the time spent outside ranging (12). In this previous study, multiple types of stimulation were provided, and thus, it was unclear which aspect (physical, visual, auditory, sensory) may have had the most impact on the pullet's development.

The aim of the current study was to assess the impacts of different types of enrichments provided throughout the rearing period on individual range use of hens across a flock cycle including the use of the length of the range when first provided access and initial stress responses of hens following first pop-hole opening. Two types of enrichments were selected, regularly replaced novel objects to simulate an unpredictable and changing environment, and structures with some opaque sides to allow perching and increased navigation within the pens. It was predicted that both types of enrichments would increase ranging behavior, that the initial range access would require adaptation by the hens to the new environment, and that the novelty hens would be best prepared for this adaptation. This study was part of an overall larger study assessing behavioral and welfare impacts of rearing enrichments in free-range hens.

## MATERIALS AND METHODS

### Ethical Statement

All research was approved by the University of New England Animal Ethics Committee (AEC17-092).

### Animals and Housing

This study used 1,386 Hy-Line® Brown layers that were reared for 16 weeks in the Rob Cumming Poultry Innovation Centre of the University of New England, Armidale, Australia, and subsequently housed in the Laureldale free-range facility of the University of New England until 65 weeks of age. Day-old chicks were obtained from a commercial supplier (including additional chicks that were not transferred to the laying facility) and placed in 9 floor-litter pens (6.2 m L x 3.2 m W) that were visually isolated via shade cloth hung on the wire pen dividers and distributed across three separate rooms. Each pen had rice hulls as ground litter, round feeders for *ad libitum* access to commercially formulated mash appropriate for different developmental stages, and water nipples. Resources were provided as per the current Australian Model Code of Practice for the Welfare of Animals—Domestic Poultry (25). The pullets were then exposed to three separate rearing enrichment treatments with one replicate of each treatment per room, balanced for location within the room. These included a control group ("control" hens) having no extra materials over the floor litter, a novelty group ("novelty" hens) where novel objects were changed at weekly intervals (e.g., balls, bottles, bricks, brooms, brushes, buckets, containers, pet toys, plastic pipes, strings, water bottles)

as well as rotated for location within the room every 3–4 days, and a structural group ("structural" hens) where four custom-designed H-shaped perching structures (L, W, H all 0.60 m) with two solid panels and one open-framed side that could be placed in different orientations were provided for the rearing duration as static enrichment. By 16 weeks of age, bird density was ∼15 kg/m$^2$ (average 174–190 pullets/pen resulting from chick mortality and some placement error). The temperature and light schedules followed the Hy-Line® Brown alternative management guidelines (26) except that the artificial LED lighting was maintained at 100 lux as the pullets were destined for outdoor access. Rooms were mechanically ventilated as needed, but no cooling system was present. Chicks were infrared beak-trimmed at the hatchery and vaccinated through rearing as per regulatory requirements and standard recommendations for the region.

At the end of rearing, 16-week-old pullets were transferred to the Laureldale free-range facility and socially remixed within pen replicates of their rearing treatment across 9 pens within a single shed (3 pen replicates per rearing treatment of similar group sizes to the rearing period). The indoor pens were of the same configuration (**Figure 1**) and visually isolated via shade cloth. Each pen contained nest boxes, perches, feeders, and water nipples to fulfill the requirements of the Australian Model Code of Practice for the Welfare of Animals—Domestic Poultry (25). Perching space was 10 cm per bird due to logistical space restrictions within the pen, but hens also perched on the tops of the feeders and waterlines. Rice hulls were placed on the floor with regular raking management and one complete litter replacement midway through the flock cycle. The LED lighting schedule gradually increased to 16 h light and 8 h dark by 30 weeks of age with an average pen light intensity of 10.0 (± 0.84 SE) lux (Lutron Light Meter, LX-112850; Lutron Electronic Enterprise CO., Ltd, Taipei, Taiwan) as measured at birds' eye height from three pen locations (front, middle, back) when the pop-holes were closed. This light intensity was the highest that could be achieved with the shed lighting system. The shed was fan-ventilated with no temperature or humidity control.

The nine indoor pens were each connected to an outdoor range area (**Figure 1**) accessible via two pop-hole openings (18 cm W × 36 cm H) and visually isolated from each other via shade cloth on the wire fences. Automatic pop-holes were first opened at 25 weeks of age (May 2018) allowing daily access to the hens for most of the daytime. The pop-holes opened at 9:15 am and closed after sunset daily. This equated to ∼9 h of available ranging time across winter followed by ∼11 h of available ranging time after daylight saving time started (October 2018 until trial completion in January 2019). The range area comprised a concrete path, followed by river rock and then a grassed area devoid of trees or additional shelters (**Figure 1**). Visual estimation from photos showed that the ranges were initially 90% covered in grass. By 8 weeks after the first range access, the grass was either destroyed by the hens or had gone through winter die-out. There was some grass regrowth in the spring (6 months after first range access) with up to 40% coverage in some pens (3 pens 0%: 1 of each treatment, 4 pens 20%: 2 novelty, 2 structural, 2 pens 40%: 2 control), but by summer (8 months after the first range access), there were only hen-resistant weeds scattered in the bare dirt. A temperature logger (Tinytag Plus 2, TGP-4500; Gemini Data Loggers Ltd, West Sussex, UK) was placed out on the range to record average daily temperature throughout the flock cycle (hourly measurements were recorded).

## Radio-Frequency Identification (RFID) System and Data

Before transfer to the laying facility, all hens were banded with microchips [Trovan® Unique ID 100 (FDX-A): operating frequency 128 kHz] glued into adjustable leg bands (Roxan Developments Ltd, Selkirk, Scotland). Radio-frequency identification (RFID) systems were set up in the indoor pens (as per (28)). These systems were designed and supported by Microchips Australia Pty Ltd (Keysborough, VIC, Australia) with equipment developed and manufactured by Dorset Identification B.V. (Aalten, the Netherlands) using Trovan® technology. Antennas were placed within the two pop-holes per pen that allowed range access, and the movement of individual hens out to the range and back inside to the pen was tracked. The RFID system recorded the date and time of each banded bird passing through the pop-hole and in which direction (onto the range, or into the pen) with a precision of 0.024 s (maximum detection velocity 9.3 m/s). Individual ranging data were collected daily from 25 until 64 weeks of age.

These daily RFID data from individual hens (272 days) were grouped into six time periods comprising 25–27, 27–31, 31–38, 38–44, 47–54, and 55–64 weeks of age. Due to technical malfunction, unforeseen circumstances, and experimental interventions (e.g., weighing days and a stressor period as part of a separate dataset), some days of data were excluded resulting in a total of 232 days analyzed across the 272-day recording period. There were 6 days of data missing for one control and one novelty pen within the 27–31 weeks recording period due to technical malfunction. Once grouped, the data were run through a custom-designed software program written in the "Delphi" language (Bryce Little, CSIRO, Agriculture and Food, St Lucia, QLD, Australia) that filtered out any unpaired or "false" readings that may occur if, for example, a hen sits inside the pop hole but does not complete a full transition onto the range or back into the pen. The same program summarized the daily data to provide an average of hours outside, the number of visits outside, the maximum individual visit time, and the total percentage of available days accessed per individual hen per age period.

## Video Recording and Data Collection

Nine Hikvision Network cameras (Model DS-2CD2232-I5 4 mm, Hikvision, Hangzhou, China) were installed to capture the range area of each pen (one camera per pen) across 14 days during pop-hole opening times excluding ∼1.2 m in front of the pop holes (due to the camera angle). Video recordings were later decoded by a single observer (blind to rearing treatment) who counted the number of hens present at different distances from the shed across the length of the range area (1.2–5, 5–10, 10–20,

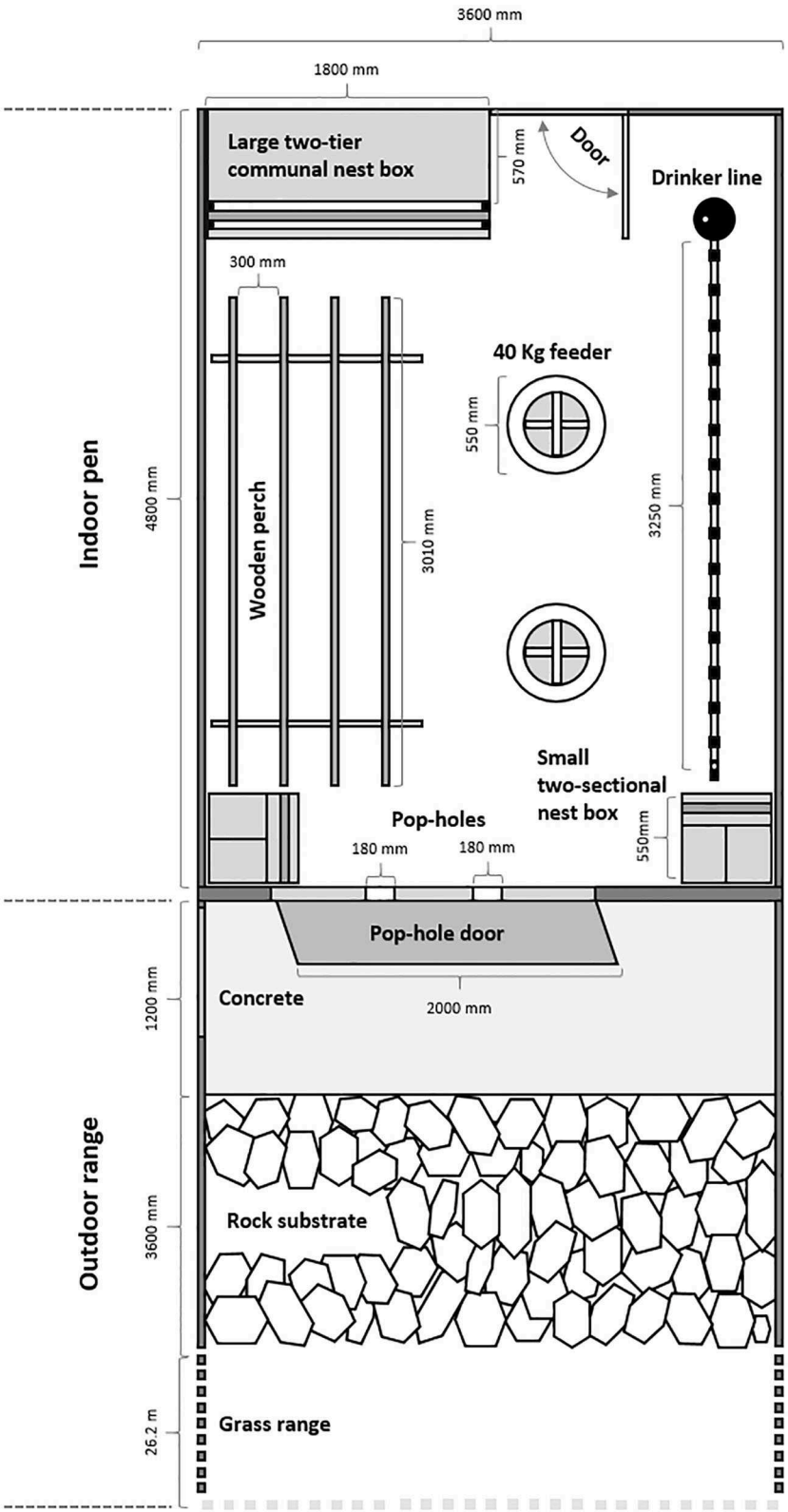


**FIGURE 1 |** Top-down view of the indoor pen and outdoor range showing placement and dimensions of the indoor perch, nest box, water, and feed resources, the range access pop-holes, and different range substrates. Each of the nine pens had identical indoor configuration except for three pens, which had a radio-frequency identification box in the front right corner that the small nest box sat upon (the small nest boxes were elevated by cinder blocks in the remaining pens). Reproduced from (27).

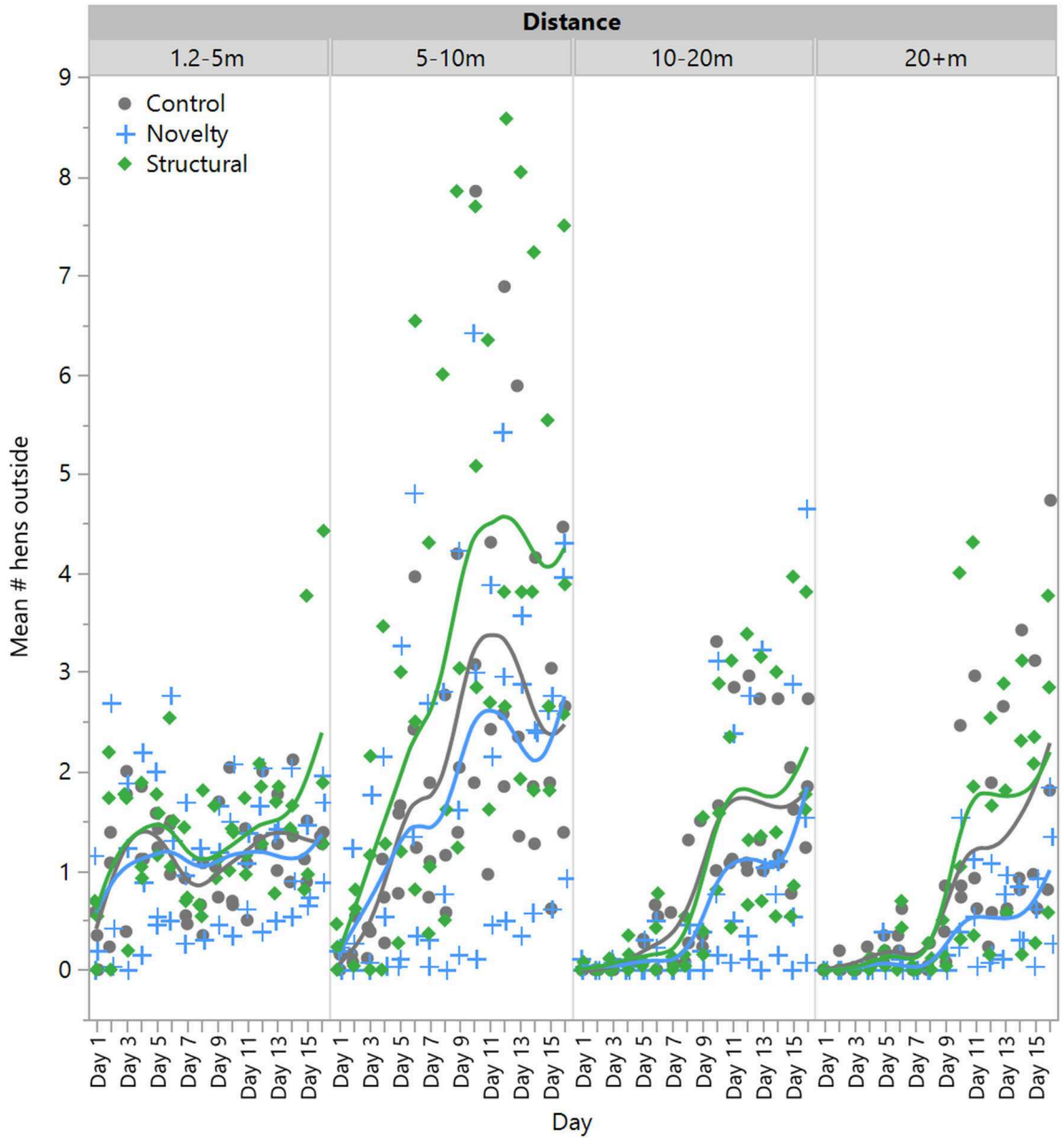


**FIGURE 2 |** The mean number of laying hens from three rearing enrichment treatments (control, novelty, structural) outside at increasing distances of the range length across the first 14 days of range access. Individual data points indicate daily means for each pen per rearing treatment.

and 20–31 m) every 30 min for the first 2 weeks of range access (total 14 days).

## Albumen Sampling

At 24 weeks of age, 4 days before the hens were provided outdoor access for the first time, a total of 50 eggs from each pen were randomly selected in the morning across all laying locations (floor, small, and large nest boxes). Substantially dirty eggs were not included. The same number of eggs was collected again 7 days following initial range access. On the day of collection, all eggs were weighed and broken open; the albumen was separated from the yolk then weighed and stored at −20°C until analysis via radioimmunoassay following the procedures reported in Downing and Bryden (29). All albumen corticosterone analyses were conducted blind to rearing treatment.

## Data and Statistical Analyses

All analyses were conducted in JMP14.0 (SAS Institute, Cary, NC, USA) with α set at 0.05. Data were checked for normality and homoscedasticity by visual inspection of the model residuals; data transformations or non-parametric tests were applied where

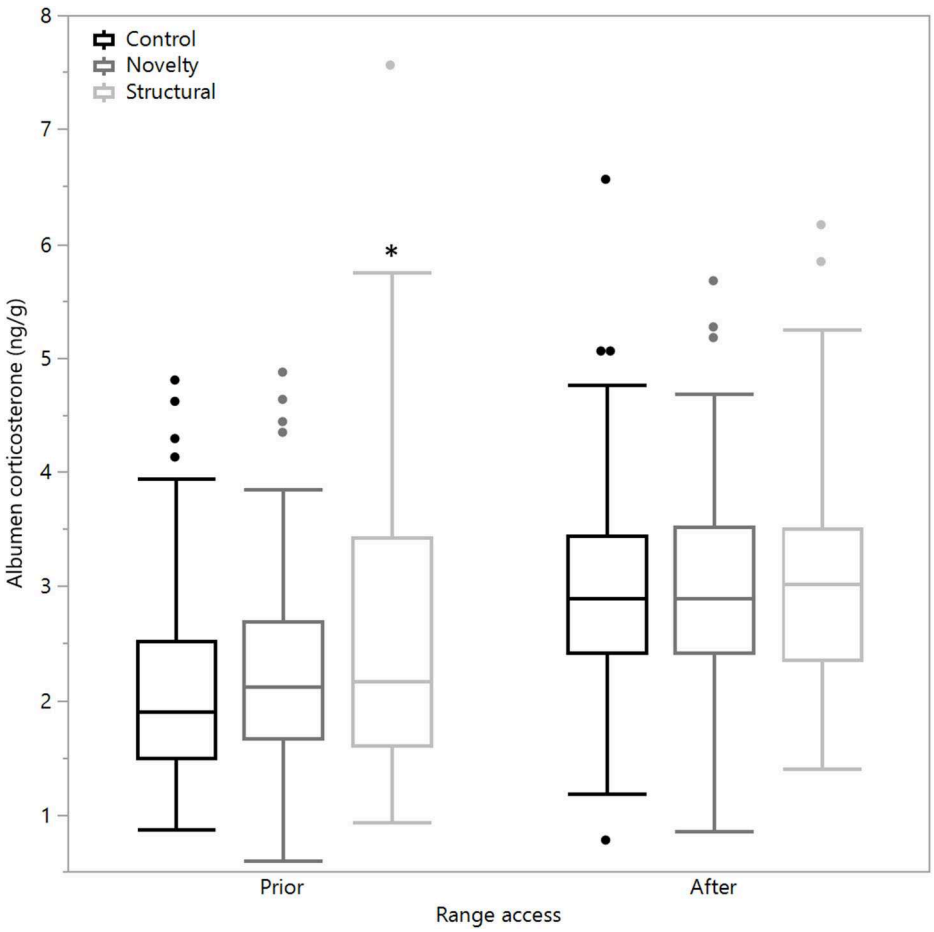


**FIGURE 3 |** The mean corticosterone concentrations (ng/g) of egg albumen from hens exposed to three rearing enrichment treatments (control, novelty, structural) sampled 4 days prior to and 7 days after the first range access. Box ends represent the first and third quartiles with whiskers extending to data within 1.5 x the interquartile range or upper and lower data points (excluding outliers) if the data do not reach the computed ranges. Isolated data points indicate outliers. The asterisk indicates that the structural hens significantly differed from the other treatment groups prior to the range access.

necessary. It was assumed that data from individual pens were independent from each other due to physical and visual separation. The video data were averaged per day per pen for each measured distance (14 days × 9 pens × 4 distances = 504 data points). Mean count values were square-root-transformed and analyzed using separate general linear mixed models (GLMMs) per distance with the fixed effects of day, rearing treatment, and their interaction and pen nested within rearing treatment included as a random effect.

The albumen corticosterone data were collated per individual sample within each treatment for prior to and after the first range access ($n = 900$). Data could not be transformed to meet assumptions of homogeneity of variance, so Kruskall–Wallis tests were applied to assess for differences between group means prior to and following range access (pen was not able to be included as a blocked effect due to unequal sample sizes). Levene's tests were applied to assess for differences in variance between treatment groups both prior to and following range access.

The RFID data of mean daily time outside (h), the mean daily number of range visits, the mean maximum individual visit time (h), and the mean proportion of available days the range was accessed were compiled per individual hen across three rearing treatments and six age periods. There was one datapoint per hen within each age period for those hens that used the range (this number increased across time as more hens started ranging). While ranging of individual hens within a pen may have been affected by other hens, we included data at the hen level as individual birds were able to be tracked. Data could not be transformed to meet assumptions of homogeneity of variance, so Kruskall–Wallis tests were applied to assess for differences between treatment groups separately for each age period. Pen was unable to be included as a blocked variable due to unequal sample sizes. Individual-bird data for an entire time period were excluded if that bird died within that specific time period. Across the flock cycle, 29 hens (2.1%) died or were removed for poor health reasons. The hours outside, the

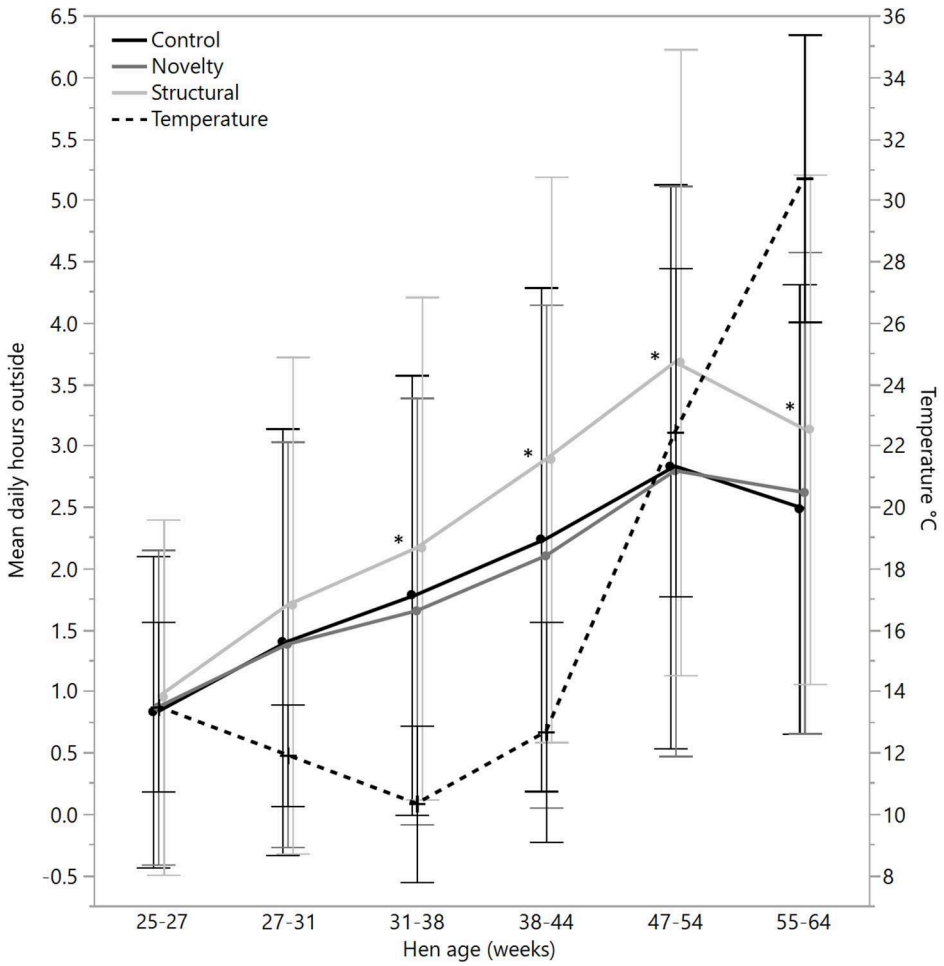


**FIGURE 4 |** The mean (± SD) daily hours spent outside on the range for hens from three rearing enrichment treatments (control, novelty, structural) across hen age periods. The mean daily temperature during ranging hours is also plotted. Asterisks indicate that the structural hens differed significantly from the control and novelty hens across four of the six age periods.

number of visits, and the proportion of available days the range was accessed were compared using Spearman's rank correlations between each successive age period, and between the first and last age periods separately for each rearing treatment. Finally, all hens that had no visits recorded on the range across the last age period were selected, and their proportion of days accessed across all previous age periods were graphed to display consistency for the most extreme indoor hens. Raw data are presented in the figures.

## RESULTS

There was no significant effect of rearing treatment on the number of hens outside at 1.2–5 m [$F_{(2,6)} = 0.23$, $P = 0.80$] or 5–10 m [$F_{(2,6)} = 0.43$, $P = 0.67$], but there was a significant effect of day [1.2–5 m: $F_{(1,132)} = 31.54$, $P < 0.0001$; 5–10 m: $F_{(1,132)} = 220.11$, $P < 0.0001$] with range use increasing across time (**Figure 2**). There was no significant interaction between rearing treatment and day [1.2–5 m: $F_{(1,132)} = 0.70$, $P = 0.50$; 5–10 m: $F_{(1,132)} = 2.35$, $P < 0.10$]. However, there was a significant interaction between rearing treatment and day for hens at 10–20 m [$F_{(2,132)} = 3.78$, $P = 0.03$] and 20+ m [$F_{(2,132)} = 5.70$, $P = 0.004$], with novelty hens showing a comparatively lower increase in the use of these farther distances across time (**Figure 2**). All hens did increase their range use across the 2-week period at these farther distances [10–20 m: $F_{(1,132)} = 318.56$, $P < 0.0001$]; 20+ m: $F_{(1,132)} = 273.38$, $P < 0.0001$, **Figure 2**.

There was a significant difference between rearing treatments prior to range access in the concentrations of albumen corticosterone ($\chi^2 = 11.03$, df = 2, $P = 0.004$) with the structural hens showing a higher corticosterone concentration as well as significantly higher variance [$F_{(2,447)} = 23.12$, $P < 0.0001$, **Figure 3**]. However, following range access, there were no differences between treatment groups in means ($\chi^2 = 0.18$, df = 2, $P = 0.92$) or variance [$F_{(2,296.33)} = 0.46$, $P = 0.63$], but all treatment groups showed elevated concentrations (**Figure 3**).

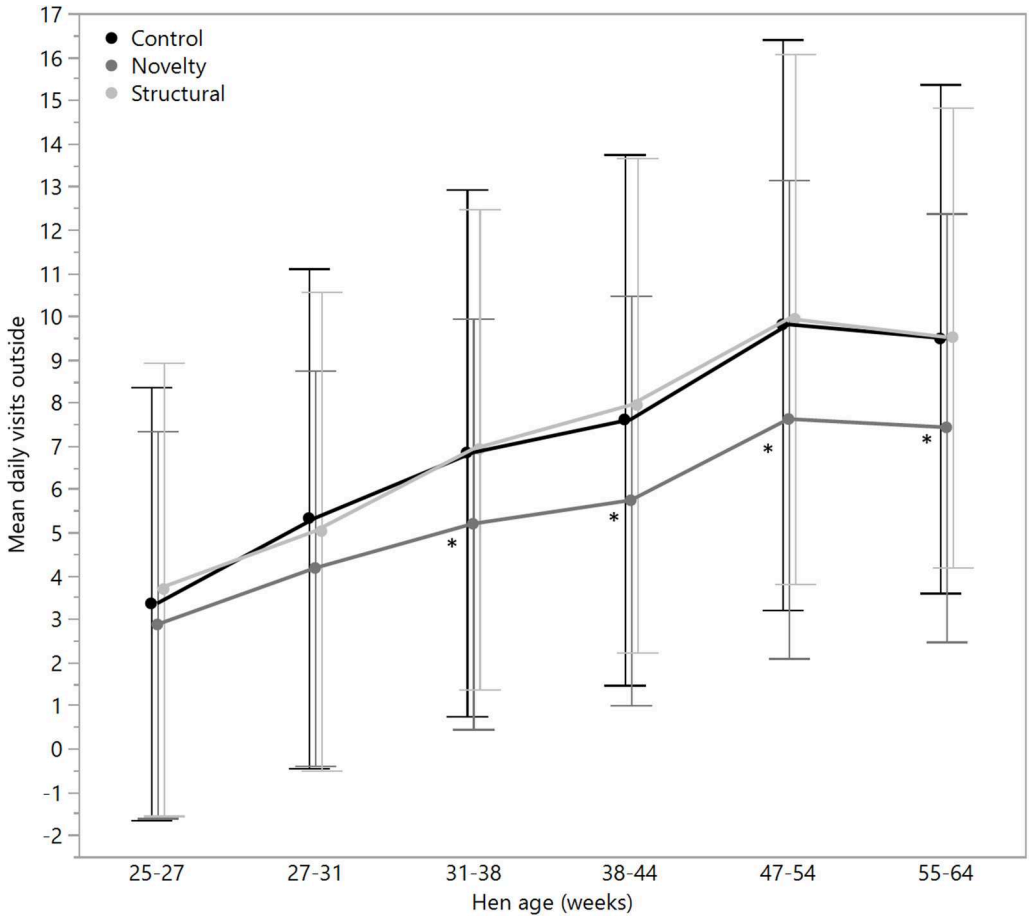


**FIGURE 5 |** The mean (± SD) daily visits to the outside range for hens from three rearing enrichment treatments (control, novelty, structural) across the periods of hen age (weeks). Asterisks indicate that the novelty hens differed significantly from the control and structural hens across four of the six age periods.

There were no significant differences between rearing treatments in the daily hours outside across the first two age points (25–27 weeks: $\chi^2 = 1.13$, df = 2, $P = 0.57$; 27–31 weeks: $\chi^2 = 2.15$, df = 2, $P = 0.34$), but there were significant differences between rearing treatments for each age period for the remainder of the flock cycle ($\chi^2 = 12.46$–$34.27$, df = 2, $P \leq 0.002$) with the hens from the structural rearing treatment spending the most time outside (**Figure 4**). There were no significant differences between rearing treatments in the number of daily visits to the range at 25–27 ($\chi^2 = 1.11$, df = 2, $P = 0.57$) and 27–31 weeks of age ($\chi^2 = 2.84$, df = 2, $P = 0.24$). For the remaining time periods, there were significant differences between rearing treatments ($\chi^2 = 22.44$–$47.20$, df = 2, $P < 0.0001$) with the novelty hens showing the fewest visits (**Figure 5**). There were significant differences between rearing treatments for the maximum visit duration across all ages ($\chi^2 = 6.37$–$54.99$, df = 2, $P \leq 0.04$), with generally the enriched hens (novelty and structural) both showing longer maximum visit times than the control hens (**Figure 6**).

There were no differences between rearing treatments in the proportion of available days that individual hens went outside at 25–27, 27–31, and 31–38 weeks ($\chi^2 = 0.02$–$4.49$, df = 2, $P \leq 0.99$), but there were differences between groups at the remaining age points ($\chi^2 = 14.40$–$23.63$, df = 2, $P \leq 0.0007$) with the structural hens spending the most days outside (**Figure 7**).

There were 98 hens that were registered with zero days outside across 55–64 weeks of age; of these, 39 hens were registered as never going outside at any point across the trial duration (control: $n = 13$, novelty: $n = 16$, structural: $n = 10$), and the remaining hens did go outside sometimes but for consistently low proportions of time (**Figure 8**).

There were correlations of 0.74 to 0.95 across adjacent age periods for daily hours, daily visits, and proportion of days outside (**Table 1**). However, there were lower correlations (0.30–0.46) for the first and last measured age periods (**Table 1**).

## DISCUSSION

This study assessed the impacts of different rearing enrichments on subsequent range use by adult hens in an experimental setting across a production cycle. Hens that were provided with perching structures including opaque sides spent the most time

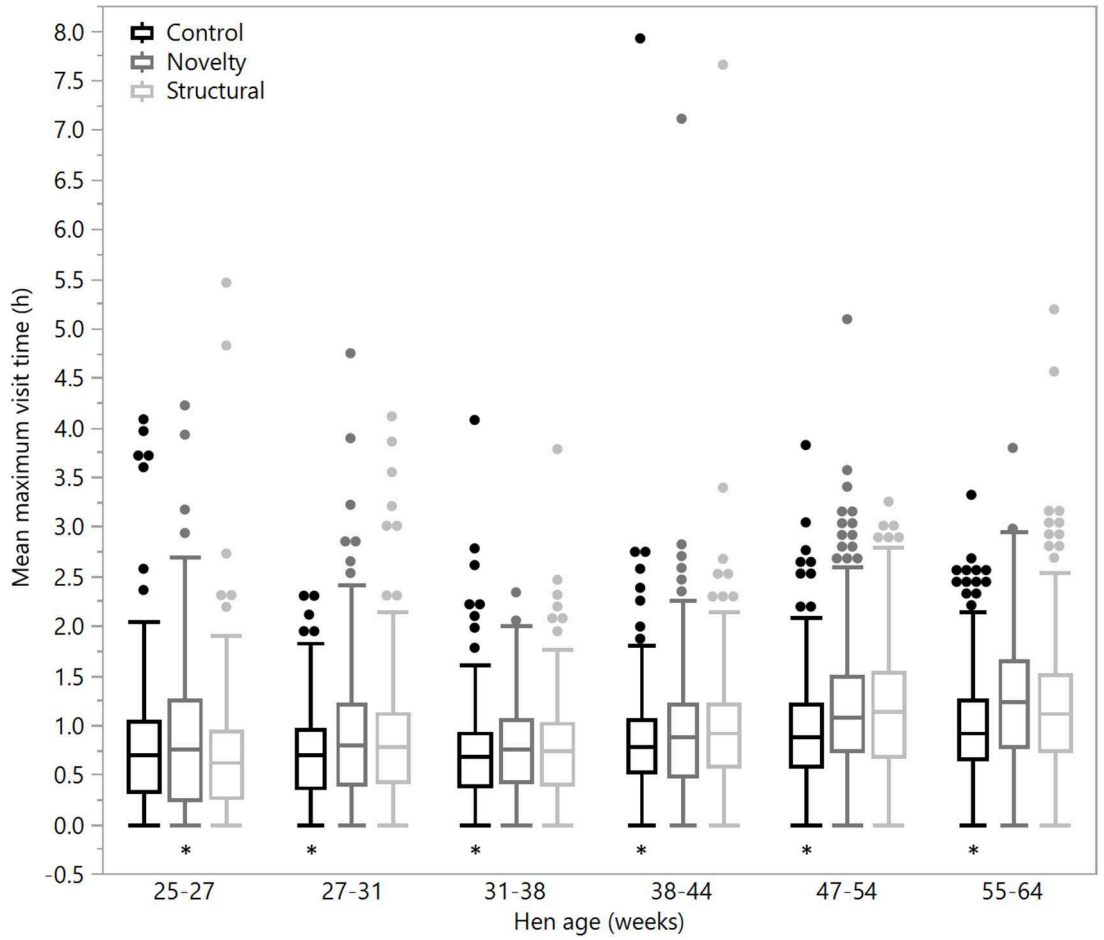


**FIGURE 6 |** The mean maximum daily visit time outside for hens from three rearing enrichment treatments (control, novelty, structural) across the periods of hen age (weeks). Box ends represent the first and third quartiles with whiskers extending to data within 1.5 x the interquartile range or upper and lower data points (excluding outliers) if the data do not reach the computed ranges. Isolated data points indicate outliers. Asterisks indicate that the control hens differed from both enriched treatment groups across five of the six age periods.

on the range, and hens that were exposed to different novel objects showed fewer visits to the range; both these enriched treatments typically supported longer individual range visit times than the control hens. There were individual differences between hens in how often they accessed the range across all rearing treatments with an increase in range use as hens aged. Most hens showed some range use by the end of the flock cycle, but a small proportion remained inside across the trial duration. Hens were slow to first use the range and showed elevated albumen corticosterone concentrations at the end of the first week. These results indicate that enrichments for pullets reared indoors can modify subsequent range use with impacts across the flock cycle.

Two types of enrichments were tested in this study that had disparate, yet sustained impacts on ranging. In a previous study that applied enrichments for the first 3 weeks of life (12), multiple types of enrichments (stimulatory and physical) were combined. These enrichments resulted in a small reduction in hours outside for the enriched hens and reduced corticosterone responses to implemented stressors. However, it was uncertain specifically what aspect of the enrichments may have had the greatest effect. The increase in ranging hours by the structural hens in the current study may have been due to improvements in their spatial navigation abilities. Previous research has shown some effects of elevated structures during rearing on the speed of completing cognitive tasks in chicks (30) or spatial jumping tasks in pullets (31). Laying hens reared in aviaries also showed improved three-dimensional use of their new pens when transferred to the laying facility compared with hens reared in cages, although these differences were not sustained past the first 4 weeks following transfer to the laying facility (32). Chicks with exposure to occlusion barriers within the first 2 weeks of development showed some modification of their spatial behavior compared with control chicks receiving no occlusion experience (33). The structural groups had experience with large opaque barriers throughout rearing, although some of the initial objects in the novelty group (cinder blocks, buckets) may have also functioned

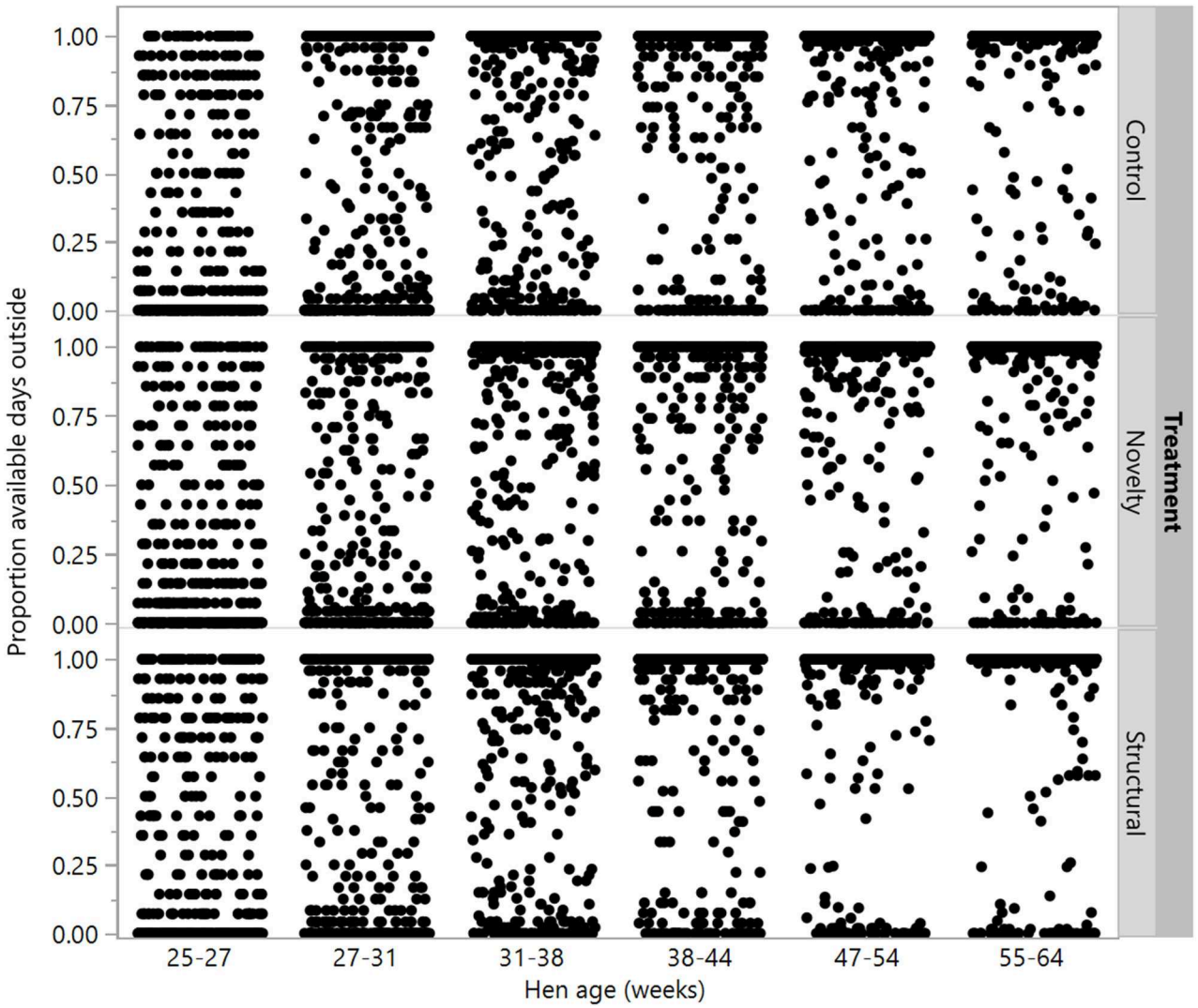


**FIGURE 7 |** The proportion of available ranging days that individual hens from three rearing treatments (control, novelty, structural) went outside across the flock cycle (25–64 weeks). Differences between treatments were found at 38–44, 47–54, and 55–64 weeks of age.

as occlusion barriers to the small chicks. The structural hens may have felt more competent in moving between the indoor and outdoor areas, thus increasing the overall amount of time they spent outdoors. However, contrary to predictions of improved spatial abilities in the structural hens, the novelty hens showed the greatest perching within the home pen upon first transfer to the layer facility at 16–17 weeks of age (34) and continued to show the highest use of the large two-tiered nest boxes (compared with small ground nest boxes or floor-laying) across the production cycle (27). The novelty hens adapted to the home pen more rapidly, which may have led to the increased time spent inside rather than out on the range once the pop-holes were opened. Finally, the structural hens may have also spent more time on the range as a result of improved social interactions. The range area would have a reduced stocking density (even at maximum occupancy) compared with the indoor pen, and more hens outside consequently would lower the indoor stocking density. Perhaps the ability to perch or move out of sight of conspecifics during rearing improved the mediation of conspecific interactions, thus improving their social spacing as adults—a hypothesis that remains to be tested.

Results indicated that in the initial period of range access, hens probably experienced a level of stress associated with exposure to a new, unfamiliar housing environment, which could account for the elevated albumen corticosterone and their hesitation to venture outside and/or use the full range area. This was anticipated given previous findings of low range use initially (12, 14) and the expectation that the outdoor environment was highly novel following a long period of indoor-only exposure. Additionally, the hens were quite old at age of first access (25 weeks), an intentional experimental decision as hens might be less adaptable at the older age, thus increasing the testing

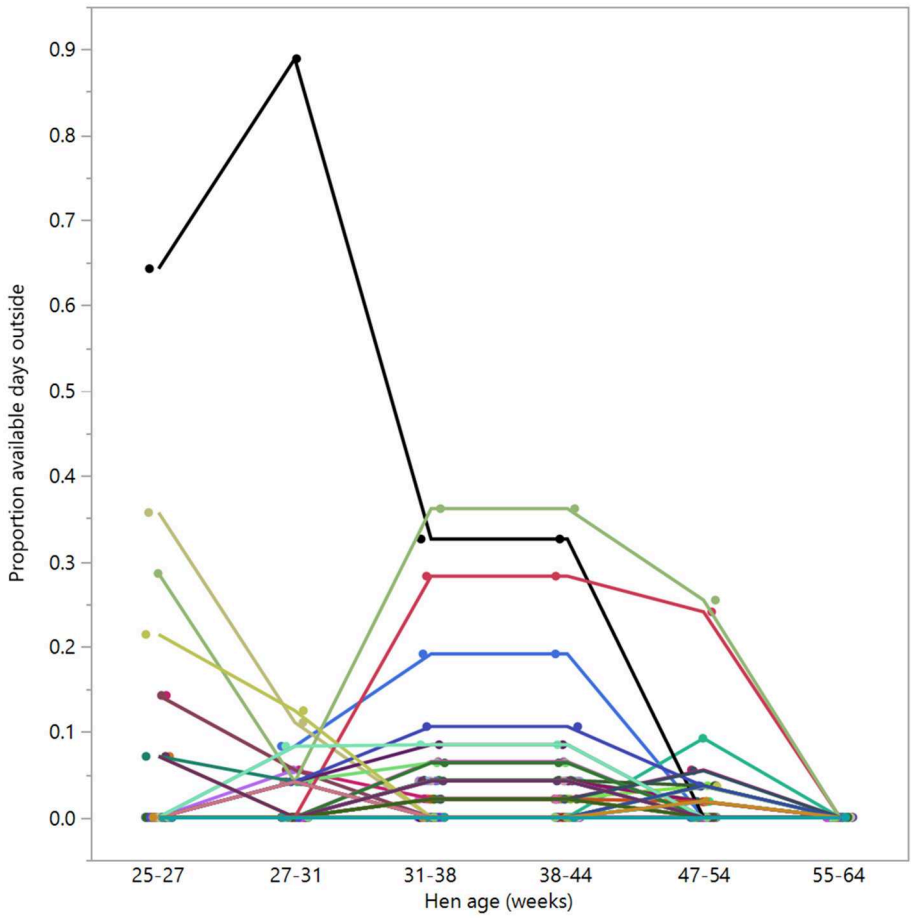


**FIGURE 8 |** The proportion of available days that the hens ($n = 98$) spent outside across different age periods. Displayed hens were selected based on showing no days outside in the last age period (55–64 weeks). Different colors represent individual hens.

stringency of any rearing enrichment effect. Alternatively, hens may have been aroused with the new experiences available to them; future tests combining valence with arousal measures would confirm the effects of initial pop-hole opening on hen affect. Contrary to expectations, the novelty hens were slowest to start using the range and travel along its full length away from the shed. The structural hens showed the smallest change in albumen corticosterone concentrations between baseline and following range access suggesting that they could have been less stressed and more capable of adapting to the range more readily. However, their mean corticosterone concentrations and variance were higher in the baseline samples compared with the other rearing treatment groups, and it is unclear why this may have been. The assay used to determine the corticosterone concentrations is a radioimmunoassay and uses antiserum that has some cross-reactivities to other steroids (29). A recent HPLC-MS-MS analysis of egg albumen reported that the corticosterone concentrations are low (35), but there was little background information provided for the hens used in the study. Comparisons of mean percentage egg production between treatment groups across 7 days prior to the baseline sampling showed some differences between the control and both enriched treatment groups (control: 87.5% production; novelty: 93.5%; structural: 93.1%), similarly across 7 days prior to the second sampling point (control: 88.3%; novelty: 91.7%; structural: 90.0%), so it is unclear to what degree cross-reactivities may have affected the corticosterone results. Other physiological measures such as blood profiles instead of or in addition to albumen corticosterone may be more informative but are difficult to measure due to the stress of handling the birds. Following the first 6 weeks of ranging, the novelty hens spent a similar amount of time on the range as the control hens, but they had fewer visits, with longer maximum durations, similar to those of the structural hens. Thus, both enrichment treatments had effects on the hens' behavior, but the mechanism of their impact is unclear. It is possible that the enrichments resulted in different degrees of brain laterality and hemispheric dominance in the hens.

A lateralized brain will improve the ability to respond to concurrent stimuli (e.g., searching for food while under threat from a predator) (36) and likely has implications for

**TABLE 1 |** The ρ values for Spearman's rank correlations between adjacent hen age periods for mean daily ranging hours, visits, and proportion of days spent outside for hens from three rearing enrichment treatments (control, novelty, structural).

| | Rearing treatment | | |
|---|---|---|---|
| **Age (weeks)** | **Control** | **Novelty** | **Structural** |
| | **Ranging hours**[a] | | |
| 25–27 and 27–31 | $\rho = 0.74$ | $\rho = 0.74$ | $\rho = 0.80$ |
| 27–31 and 31–38 | $\rho = 0.82$ | $\rho = 0.81$ | $\rho = 0.81$ |
| 31–38 and 38–44 | $\rho = 0.92$ | $\rho = 0.91$ | $\rho = 0.92$ |
| 38–44 and 47–54 | $\rho = 0.92$ | $\rho = 0.90$ | $\rho = 0.92$ |
| 47–54 and 55–64 | $\rho = 0.94$ | $\rho = 0.95$ | $\rho = 0.95$ |
| *25–27 and 55–64* | *$\rho = 0.38$* | *$\rho = 0.36$* | *$\rho = 0.46$* |
| | **Ranging visits**[a] | | |
| 25–27 and 27–31 | $\rho = 0.75$ | $\rho = 0.76$ | $\rho = 0.80$ |
| 27–31 and 31–38 | $\rho = 0.84$ | $\rho = 0.81$ | $\rho = 0.81$ |
| 31–38 and 38–44 | $\rho = 0.93$ | $\rho = 0.89$ | $\rho = 0.89$ |
| 38–44 and 47–54 | $\rho = 0.88$ | $\rho = 0.87$ | $\rho = 0.88$ |
| 47–54 and 55–64 | $\rho = 0.90$ | $\rho = 0.90$ | $\rho = 0.88$ |
| *25–27 and 55–64* | *$\rho = 0.37$* | *$\rho = 0.33$* | *$\rho = 0.46$* |
| | **Proportion days ranging**[a] | | |
| 25–27 and 27–31 | $\rho = 0.81$ | $\rho = 0.77$ | $\rho = 0.83$ |
| 27–31 and 31–38 | $\rho = 0.84$ | $\rho = 0.82$ | $\rho = 0.86$ |
| 31–38 and 38–44 | $\rho = 0.81$ | $\rho = 0.82$ | $\rho = 0.83$ |
| 38–44 and 47–54 | $\rho = 0.84$ | $\rho = 0.85$ | $\rho = 0.74$ |
| 47–54 and 55–64 | $\rho = 0.79$ | $\rho = 0.80$ | $\rho = 0.80$ |
| *25–27 and 55–64* | *$\rho = 0.34$* | *$\rho = 0.31$* | *$\rho = 0.30$* |

*A comparison between the first and last age periods for each ranging variable is italicized.*
[a]*All $P < 0.0001$.*

animal welfare such as the display of a negative bias or an elevated response to stressful situations (37, 38). The left hemisphere controls established behavioral patterns compared with the right hemisphere that attends to unexpected stimuli; an overview of the hemispheric specializations is provided in Rogers (36) and Rogers and Kaplan (38). The different types of enrichments provided may have either improved the degree of hemispheric flexibility and how the hens react to stimuli and their surrounding environment (38), or increased the dominance of a specific hemisphere thus altering the main hemisphere attending to the environment. Changes in cellular neural processes following enrichment have been demonstrated in rodents including differences based on the period of exposure (39). Thus, confirmation of the impacts of different types of enrichments on laterality and neural pathways warrants further investigation, particularly the optimal timing of enrichment exposure in pullets (23).

The number of hens outside and the time spent outside generally increased with age indicating acclimation to the range area, but there was a drop at the end of the production cycle, which may have resulted from the increasing summer temperatures (see **Figure 4**). Across all rearing treatments, there were clear individual differences in the degree of time hens spent ranging, which is further confirmation to the findings of multiple previous studies [e.g., (9, 12, 28, 40)]. This individual variation was present across all rearing treatments indicating that no specific rearing environment eliminated variability in ranging patterns between hens. The effectiveness of provided enrichments could be impacted by the degree of interaction that each hen specifically had with the enrichment objects and/or their perception of them (i.e., stimulating, stressful, benign). The correlations of range use between successive age periods indicate consistency in individual ranging patterns, which was found to have implications for some welfare measures of the hens in this study, although most hens were in relatively good condition at the end of the trial (41). Across the whole trial, ∼3% of the hens never went outside. These likely represent an extreme end of the population distribution and may be related to differences in affective states with more fear and anxiety in some hens leading them to remain indoors within a free-range system (16). A free-range system that provides a choice of different environments may thus be conducive to catering for individual differences in welfare needs (42).

## CONCLUSION

Providing range access during rearing may improve range access as adults, but this is not a feasible strategy across all countries. Rearing enrichments may be an alternative to improve an adult hen's use of the range. Different types of enrichments can have varying impacts on ranging behavior, where in the current study, stable perching structures with opaque sides provided during rearing led to the highest use of the range area in adult hens. The mechanism of impact may have been through changes in brain lateralization, but further studies would be needed to test this hypothesis.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

DC and CL contributed to the conception and design of the study. DC, TD, JD, and AC-B conducted the experiments. DC performed the statistical analyses and wrote the first draft of the manuscript. DC, JD, and CL revised the manuscript. DC and CL acquired funding for the research. All authors contributed to manuscript revision, read and approved the submitted version.

## FUNDING

Funding support was provided by Poultry Hub Australia (grant 2017-20).

## ACKNOWLEDGMENTS

We are grateful to V. Uthappa (Microchips Australia Pty Ltd, Keysborough, VIC, Australia) for technical support on the RFID system. Thank you to J. Lea, S. Belson, T. Kalinowski, D. Niemeyer, and B. Niemeyer (CSIRO), and to E. Jurecky, J. Whitten, N. Pearce, E. Dickson, and M.S. Bari (University of New England) for data collection, technical, and husbandry assistance.

## REFERENCES

1. Carey R, Parker C, Scrinis G. Capturing the meaning of 'free range': The contest between producers, supermarkets and consumers for the higher welfare egg label in Australia. *J Rural Stud.* (2017) 54:266–75. doi: 10.1016/j.jrurstud.2017.06.014
2. Bray HJ, Ankeny RA. Happy chickens lay tastier eggs: motivations for buying free-range eggs in Australia. *Anthrozoös*. (2017) 30:213–26. doi: 10.1080/08927936.2017.1310986
3. Chielo LI, Pike T, Cooper J. Ranging behaviour of commercial free-range laying hens. *Animals*. (2016) 6:28. doi: 10.3390/ani6050028
4. Campbell DLM, Hinch GN, Downing JA, Lee C. Outdoor stocking density in free-range laying hens: effects on behaviour and welfare. *Animal*. (2017) 11:1036–45. doi: 10.1017/S1751731116002342
5. Rodriguez-Aurrekoetxea A, Estevez I. Use of space and its impact on the welfare of laying hens in a commercial free-range system. *Poultr Sci.* (2016) 95:2503–13. doi: 10.3382/ps/pew238
6. Diep AT, Larsen H, Rault J-L. Behavioural repertoire of free-range laying hens indoors and outdoors, and in relation to distance from the shed. *Aus Vet J.* (2018) 96:127–31. doi: 10.1111/avj.12684
7. Pettersson IC, Freire R, Nicol CJ. Factors affecting ranging behaviour in commercial free-range hens. *World's Poultr Sci J.* (2016) 72:137–49. doi: 10.1017/S0043933915002664
8. De Koning C, Kitessa SM, Barekatain R, Drake K. Determination of range enrichment for improved hen welfare on commercial fixed-range free-range layer farms. *Animal Product Sci.* (2018) 59:1336–48. doi: 10.1071/AN17757
9. Richards GJ, Wilkins LJ, Knowles TG, Booth F, Toscano MJ, Nicol CJ, et al. Continuous monitoring of pop hole usage by commercially housed free-range hens throughout the production cycle. *Vet Rec.* (2011) 169:338. doi: 10.1136/vr.d4603
10. Nagle TAD, Glatz PC. Free range hens use the range more when the outdoor environment is enriched. *Asian-Aus J Animal Sci.* (2012) 25:584–91. doi: 10.5713/ajas.2011.11051
11. Pettersson IC, Weeks CA, Nicol CJ. Provision of a resource package reduces feather pecking and improves ranging distribution on free-range layer farms. *Appl Animal Behav Sci.* (2017) 195:60–6. doi: 10.1016/j.applanim.2017.06.007
12. Campbell DLM, Hinch GN, Downing JA, Lee C. Early enrichment in free-range laying hens: effects on ranging behaviour, welfare and response to stressors. *Animal*. (2018) 12:575–84. doi: 10.1017/S1751731117001859
13. Bestman M, Verwer C, van Niekerk T, Leenstra F, Reuvekamp B, Amsler-Kapalaite Z, et al. Factors related to free-range use in commercial laying hens. *Appl Animal Behav Sci.* (2019) 214:57–63. doi: 10.1016/j.applanim.2019.02.015
14. Gilani A-M, Knowles TG, Nicol CJ. Factors affecting ranging behaviour in young and adult laying hens. *Br Poultr Sci.* (2014) 55:127–35. doi: 10.1080/00071668.2014.889279
15. Campbell DLM, Hinch GN, Downing JA, Lee C. Fear and coping styles of outdoor-preferring, moderate-outdoor and indoor-preferring free-range laying hens. *Appl Animal Behav Sci.* (2016) 185:73–7. doi: 10.1016/j.applanim.2016.09.004
16. Campbell DLM, Dickson EJ, Lee C. Application of open field, tonic immobility, and attention bias tests to hens with different ranging patterns. *PeerJ*. (2019) 7:e8122. doi: 10.7717/peerj.8122
17. Hartcher KM, Hickey KA, Hemsworth PH, Cronin GM, Wilkinson SJ, Singh M. Relationships between range access as monitored by radio frequency identification technology, fearfulness, and plumage damage in free-range laying hens. *Animal.* (2016) 10:847–53. doi: 10.1017/S1751731115002463
18. Janczak AM, Riber AB. Review of rearing-related factors affecting the welfare of laying hens. *Poultr Sci.* (2015) 94:1454–69. doi: 10.3382/ps/pev123
19. Widowski T, Torrey S. Rearing young birds for adaptability. In: Mench JA, editor. *Advances in Poultry Welfare*. Duxford: Woodhead Publishing Group (2018). pp. 49–77.
20. Tahamtani FM, Hansen TB, Orritt R, Nicol C, Moe RO, Janczak AM. Does rearing laying hens in aviaries adversely affect long-term welfare following transfer to furnished cages? *PLoS ONE.* (2014) 9:e107357. doi: 10.1371/journal.pone.0107357
21. Leenstra F, Maurer V, Galea F, Bestman M, Amsler-Kepalaite Z, Visscher J, et al. Laying hen performance in different production systems; why do they differ and how to close the gap? *Eur Poultr Sci.* (2014) 78. doi: 10.1399/eps.2014.53
22. Newberry RC. Environmental enrichment: increasing the biological relevance of captive environments. *Appl Anim Behav Sci.* (1995) 44:229–43. doi: 10.1016/0168-1591(95)00616-Z
23. Campbell DLM, de Haas EN, Lee C. A review of environmental enrichment for laying hens during rearing in relation to their behavioral and physiological development. *Poultr Sci.* (2019) 98:9–28. doi: 10.3382/ps/pey319
24. Campbell DLM, Horton BJ, Hinch GN. Using radio-frequency identification technology to measure synchronised ranging of free-range laying hens. *Animals*. (2018) 8:210. doi: 10.3390/ani8110210
25. Primary Industries Standing Committee. *Model Code of Practice for the Welfare of Animals – Domestic Poultry, 4th Edition*. Collingwood, VIC: CSIRO Publishing (2002).
26. Hy-Line®. *Hy-Line® Brown Management Guide for Alternative Systems-Australia*. (2016). Available online at: https://www.hyline.com/userdocs/pages/BRN_ALT_COM_AUS.pdf (accessed November 1, 2017).
27. Bari MS, Cohen-Barnhouse AM, Campbell DLM. Early rearing enrichments influenced nest use and egg quality in free-range laying hens. *Animal.* (2020) 14:1249–57. doi: 10.1017/S1751731119003094
28. Campbell DLM, Hinch GN, Dyall TR, Warin L, Little BA, Lee C. Outdoor stocking density in free-range laying hens: radio-frequency identification of impacts on range use. *Animal.* (2017) 11:121–30. doi: 10.1017/S1751731116001154
29. Downing JA, Bryden WL. Determination of corticosterone concentrations in egg albumen: A non-invasive indicator of stress in laying hens. *Physiol Behav*. (2008) 95:381–7. doi: 10.1016/j.physbeh.2008.07.001
30. Norman KI, Adriaense JEC, Nicol CJ. The impact of early structural enrichment on spatial cognition in layer chicks. *Behav Proces.* (2019) 164:167–74. doi: 10.1016/j.beproc.2019.05.008
31. Gunnarsson S, Yngvesson J, Keeling LJ, Forkman B. Rearing without early access to perches impairs the spatial skills of laying hens. *Appl Animal Behav Sci.* (2000) 67:217–28. doi: 10.1016/S0168-1591(99)00125-2
32. Brantsæter M, Nordgreen J, Rodenburg TB, Tahamtani FM, Popova A, Janczak AM. Exposure to increased environmental complexity during rearing reduces fearfulness and increases use of three-dimensional space in laying hens (*Gallus gallus domesticus*). *Front Vet Sci.* (2016) 3:14. doi: 10.3389/fvets.2016.00014
33. Freire R, Cheng H-W, Nicol CJ. Development of spatial memory in occlusion-experienced domestic chicks. *Animal Behav.* (2004) 67:141–50. doi: 10.1016/j.anbehav.2003.03.015
34. Campbell DLM, Gerber PF, Downing JA, Lee C. Rearing enrichments had minimal effects on pullet behavior and welfare. *Animals*. (2020) 10:314. doi: 10.3390/ani10020314
35. Caulfield MP, Padula MP. HPLC MS-MS analysis shows measurement of corticosterone in egg albumen is not a valid indicator of chicken welfare. *Animals*. (2020) 10:821. doi: 10.3390/ani10050821

36. Rogers LJ. The two hemispheres of the avian brain: their differing roles in perceptual processing and the expression of behaviour. *J Ornithol.* (2012) 153:S61–74. doi: 10.1007/s10336-011-0769-z
37. Rogers LJ. Relevance of brain and behavioural lateralization to animal welfare. *Appl Animal Behav Sci.* (2010) 127:1–11. doi: 10.1016/j.applanim.2010.06.008
38. Rogers LJ, Kaplan G. Does functional lateralization in birds have any implications for their welfare? *Symmetry*. (2019) 11:1043. doi: 10.3390/sym11081043
39. Ohline SM, Abraham WC. Environmental enrichment effects on synaptic and cellular physiology of hippocampal neurons. *Neuropharmacology.* (2019) 145:3–12. doi: 10.1016/j.neuropharm.2018.04.007
40. Larsen H, Cronin GM, Gebhardt-Henrich SG, Smith CL, Hemsworth PH, Rault JL. Individual ranging behaviour patterns in commercial free-range layers as observed through RFID tracking. *Animals*. (2017) 7:21. doi: 10.3390/ani7030021
41. Bari MS, Laurenson YCSM, Cohen-Barnhouse AM, Walkden-Brown SW, Campbell DLM. Effects of outdoor ranging on external and internal health parameters for hens from different rearing enrichments. *PeerJ*. (2020) 8:e8720. doi: 10.7717/peerj.8720
42. Richter SH, Hintze S. From the individual to the population – and back again? Emphasising the role of the individual in animal welfare science. *Appl Animal Behav Sci.* (2019) 212:1–8. doi: 10.1016/j.applanim.2018.12.012

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 August 2020
doi: 10.3389/fvets.2020.00587

# Cell Proliferation in the Adult Chicken Hippocampus Correlates With Individual Differences in Time Spent in Outdoor Areas and Tonic Immobility

*Elena A. Armstrong*[1,2*†], *Bernhard Voelkl*[3], *Sabine Voegeli*[3], *Sabine G. Gebhardt-Henrich*[3], *Jonathan H. Guy*[1,4], *Victoria Sandilands*[5], *Tim Boswell*[1,4], *Michael J. Toscano*[3] *and Tom V. Smulders*[1,2]

[1] *Centre for Behaviour and Evolution, Newcastle University, Newcastle upon Tyne, United Kingdom,* [2] *Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom,* [3] *Centre for Proper Housing: Poultry and Rabbits (ZTHZ), University of Bern, Bern, Switzerland,* [4] *School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom,* [5] *Department of Agriculture, Horticulture, and Engineering Science, SRUC, Edinburgh, United Kingdom*

OPEN ACCESS

**Edited by:**
*Jean-Loup Rault, University of Veterinary Medicine Vienna, Austria*

**Reviewed by:**
*Ludovic Calandreau, Institut National de Recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), France*
*Peta S. Taylor, University of New England, Australia*

***Correspondence:**
*Elena A. Armstrong*
*e.a.armstrong@newcastle.ac.uk*

**†ORCID:**
*Elena A. Armstrong*
*orcid.org/0000-0003-1734-4363*

**Specialty section:**
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

**Received:** *12 March 2020*
**Accepted:** *21 July 2020*
**Published:** *26 August 2020*

**Citation:**
*Armstrong EA, Voelkl B, Voegeli S, Gebhardt-Henrich SG, Guy JH, Sandilands V, Boswell T, Toscano MJ and Smulders TV (2020) Cell Proliferation in the Adult Chicken Hippocampus Correlates With Individual Differences in Time Spent in Outdoor Areas and Tonic Immobility. Front. Vet. Sci. 7:587. doi: 10.3389/fvets.2020.00587*

Access to outdoor areas is provided as a means of enhancing welfare in commercial systems for laying hens (*Gallus gallus domesticus*), but substantial individual differences exist in their proportional use. Baseline cell proliferation levels of Adult Hippocampal Neurogenesis (AHN) have been associated with individual differences in reactive vs. proactive coping style, and in both mammals and birds, AHN is upregulated by positive experiences including environmental enrichment and exercise. We thus sought to explore whether individual differences in use of outdoor areas and in tonic immobility responses (indicative of fearfulness) were associated with hippocampal cell proliferation and neuronal differentiation. Radio frequency identification technology was used to track the ranging behavior of 440 individual focal hens within a commercially-relevant system over a 72-days period, after which tonic immobility durations were measured. Following hippocampal tissue collection from 58 focal hens, proliferation and neuronal differentiation were measured through quantitative PCR for proliferating cell nuclear antigen (PCNA) and doublecortin mRNA, respectively. Individual differences in tonic immobility duration positively correlated with PCNA expression over the whole hippocampal formation, while greater time spent in outdoor areas (the grassy range and stone yard) was associated with higher proliferation in the rostral subregion. Basal proliferation in the chicken hippocampal formation may thus relate to reactivity, while levels in the rostral region may be stimulated by ranging experience. Doublecortin expression in the caudal hippocampus negatively co-varied with time on the grassy range, but was not associated with tonic immobility duration. This suggests that ranging outside may be associated with stress. Within laying hen flocks, individual differences in hippocampal plasticity thus relate to coping style and use of external areas.

**Keywords: hippocampal formation, avian brain, adult neurogenesis, free-range laying hens, individual differences, animal welfare, *Gallus gallus domesticus***

## INTRODUCTION

Within commercial flocks of laying hens, variation between individuals may be associated with differing experience and overall welfare. For example, many systems provide access to outdoor areas as a form of enrichment, which expands freedom of movement, behavioral repertoire, exploration, and foraging opportunities for hens, beyond those already afforded by the barns (1, 2). Outdoor ranges also offer an environment of greater unpredictability than the barn interior, where conditions are tightly controlled (3). However, there is substantial variation in the extent to which individual hens use these external areas. Use of radio frequency identification (RFID) tracking within flocks consistently highlights distinct subgroups, wherein a proportion of hens access the range daily, while others seldom or never venture outside (4–7). Factors underlying this variation in ranging propensity are not yet understood, but may relate to aspects of personality, defined as consistent inter-individual differences in behavior (8).

A well-characterized dimension of animal personality is the tendency to adopt an active (or proactive) vs. passive (or reactive) behavioral strategy when challenged (9), also referred to as a coping style (10). Reactive/passive individuals are predisposed toward displaying a freezing-type fear response as opposed an active fight or flight response (11), and are thus more easily induced into immobility and remain in this state longer (9, 12). Consequently, individual differences in reactivity for hens may be reflected in their durations of tonic immobility (TI): a catatonic-like freezing response induced by brief physical restraint in an upturned position (13). Consistent with a personality trait, variation in duration of the TI response is heritable (14, 15). In line with freezing less, proactive individuals are more prone to exploration (16, 17).

Behavioral strategy also relates to individual differences in speed vs. accuracy during learning, with proactive individuals acquiring simple novel tasks more quickly (18, 19). For example, black-capped chickadees (*Poecile atricapillus*) that readily enter a novel environment are faster to learn an acoustic discrimination task (20). In hens, proactive behavior has been shown to predict predisposition to use the outdoor range. When tested before any range access, pullets from an enriched rearing environment that were quickest to reach T-maze success (presumably pro-active) also proceeded to visit the range most frequently over the 4 successive weeks (3). However, while proactive individuals tend to maintain rigid, routine-like behavior, reactive individuals are more sensitive to changes in the environment/task requirements and display enhanced behavioral flexibility (10, 21, 22). Rats selectively bred for their ability to learn new configurations in a maze task were more susceptible to TI and slower to explore a novel environment than those bred for low maze performance (23). In birds, more explorative adult red junglefowl (19) and black-capped chickadees (24) are slower at reversal learning, while behavioral flexibility is positively correlated with fearfulness in junglefowl chicks (25). Moreover, low ranging broiler chickens improved in accuracy of spatial discrimination between trials of a memory task occurring on the same day, whereas higher rangers behaved inflexibly and did not alter their performance on the second trial (26). Compared to higher rangers, chickens that ranged less were also better at inhibiting their behavior by detouring to the sides of a transparent cylinder to access a food reward, rather than pecking the cylinder walls (27).

Such individual differences in behavior may be reflected by variation in neural plasticity. Plasticity has been defined as the reciprocal interaction between brain structure and function, and forms the neurobiological basis of individuality (28). A site of notable post-developmental plasticity in the mammalian brain is the hippocampus, wherein new neurons continue to be produced and functionally integrated into the dentate gyrus subfield (29–32) through a process called Adult Hippocampal Neurogenesis (AHN). AHN has several stages: (i) proliferation of progenitor cells; (ii) migration and neural differentiation; (iii) maturation of immature neurons; and (iv) functional integration of new mature neurons into the pre-existing neural circuitry (33, 34). The various stages of AHN can be quantified using different markers. Proliferating cell nuclear antigen (PCNA) is expressed by actively dividing cells (35), while doublecortin (DCX) is a microtubule-associated protein expressed by both proliferative (type-2b &—3) and post-mitotic differentiating neurons (33).

Interestingly, behavioral strategy is reflected in levels of proliferation in the hippocampus. Quantitative PCR indicated that Atlantic salmon (*Salmo salar*) characterized as reactive had a higher basal expression of PCNA mRNA in the hippocampal homolog than their proactive conspecifics (36). Furthermore, cell proliferation in rats that predominantly responded to a novel environment by freezing was twice that observed in proactive rats, while proliferating cell numbers positively correlated with durations of freezing on an individual level (37). The rigid and inflexible behavior displayed by proactive individuals has recently been linked to limitations in their neural plasticity (38). A causal role of newborn cells in flexible spatial behavior has been demonstrated through experimental suppression of neurogenesis. Mice with experimentally ablated AHN are impaired in learning a changed (reversed) goal location in a water maze (39), and in avoiding a rotating shock zone when this is added to a stationary zone learnt first (40). As such, an association between proliferation in the hippocampus and behavioral strategy may relate to the requirement of plasticity for flexible spatial behavior.

AHN is also sensitive to the environment and modulated by long-term experience. In the mammalian brain, AHN is stimulated by experiences associated with positive affect, including environmental enrichment (41), voluntary running exercise (42) and antidepressant treatment (43, 44), but suppressed by various forms of chronic negative stress [e.g., (45, 46)]. In line with a functional gradient across the longitudinal axis of the mammalian hippocampus (47), enrichment (48, 49), and exercise (50) preferentially upregulate AHN in the dorsal mouse dentate gyrus, while chronic stress suppresses it in the ventral region (51). The increase in AHN due to environmental enrichment is also typically accompanied by a decrease in anxiety-like behavior in mice (52).

Though the avian hippocampal formation (HF) differs from the mammalian structure in cytoarchitecture and notably lacks a dentate gyrus, they are homologous, and functional similarities are evident in domains including navigation, spatial memory, and modulation of the glucocorticoid stress response (53–55). AHN levels in birds are similarly stimulated by environmental enrichment and complexity (56, 57), but downregulated by sources of chronic stress, including captivity, food restriction, constant light, unpredictable chronic mild stress, and keel bone fractures (58–62). A homologous functional gradient may also exist in the avian HF (63, 64), wherein the rostral subregion is equivalent to the dorsal rodent region and the caudal avian region is the ventral rodent homolog.

In the present study, RFID tags were used to track individual ranging behavior in terms of the proportional time that hens spent in four distinct areas: (1) the barn, (2) an adjoining covered wintergarden, (3) an adjacent uncovered stone yard, and (4) a large, grassy range. This set-up may facilitate separation of the implications that various aspects of the environment have for hen behavior and AHN. For example, the wintergarden provides fresh air but cover from rain, both the stone yard and range are exposed to the elements and to predators, and the range alone provides grass. Ranging a greater distance from the barn has been positively associated with welfare parameters in broiler chickens (65). At the end of the study, TI durations were measured and hippocampal expression of PCNA and DCX was quantified. Our research group has previously established that transcription of the DCX gene in the mouse hippocampus reflects DCX-immunoreactive cell densities under control and enriched housing conditions (49).

If individual differences in AHN relate to personality type, we would predict pro-active hens with shorter TI times to be more likely to explore the range, and AHN should co-vary negatively with ranging and positively with TI. However, if ranging experience upregulates AHN while reducing anxiety, individual differences in AHN should correlate positively with time on the range but negatively with durations of TI. Based upon putative subregional specialization in the HF, in the latter scenario, cognitive enrichment arising from broader ranging would be predicted to correlate most strongly with AHN in the rostral HF, while a negative relationship between anxiety (TI) and AHN may be evident especially in the caudal region. In the former scenario, AHN should correlate positively with TI time throughout the entire HF. This work represents a first exploration of the potential associations between hippocampal plasticity, ranging behavior and coping style in domestic chickens.

# MATERIALS AND METHODS

## Animals and Facilities

Experimental use of the animals was approved by the Bern Kantonal Authority (BE-46/16) and the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549), and procedures complied with Swiss regulations regarding their treatment. Standard commercial protocols were followed, including *ad-libitum* access to food and water. Following on-site rearing [detailed in (66)], 17 weeks old Brown Nick (H&N International) laying hens were transferred to a commercial laying hen house at the Aviforum (Zollikofen, Switzerland). Only one of the barn's two halves was used for the present study, wherein pens were equipped with a system that allowed the tracking of individual animals. The four study pens (each 12.9 $m^2$) contained a Rihs Bolegg II commercial aviary system (Krieger AG, Ruswil, Switzerland) with a stocking density of 9.33 hens/$m^2$. The aviary structure and group nests lined one wall, and the floor of the barn was covered with 10 cm of wood shavings. The aviary was 2.40 m high and consisted of three tiers, with integrated equipment comprising: a manure belt, feeding chain, and nipple drinkers within the lowest tier; a manure belt within the middle tier; and a feeding chain and nipple drinkers within the highest tier. Plastic mushroom-shaped perches were provided on the lowest and highest tiers and plastic platforms to move between tiers were provided along both aviary sides (30 cm in width and at 70 cm height from the floor). Nest entries were square plastic grids (size 2.5 × 5 cm). External to the barn were three separate areas: a wintergarden, stone yard and grassy range, each linked at a single location (pophole or gate) to facilitate sequential movement of birds when open, but closed to limit access as required by the management protocol. Fencing between pens maintained divided populations within all (internal and external) areas. Adjacent to the barn interior, birds had access to the winter-garden (∼17.55 $m^2$ per pen), which was entirely covered by a solid roof and surrounded by wire mesh on the sides and in between pens. The floor of the winter-garden was lined with a thin layer of wood shavings of the same type provided within the barn, and the area was equipped with nipple drinkers and perches. A manually-operated pophole separated the winter-garden from an uncovered yard area (∼88 $m^2$ per pen) which was lined with small stones and enclosed by a fence. Beyond a gate in the fence surrounding the stone yard was the "free-range:" an open, grassy pasture with an average size of 288 $m^2$ per pen. The grass was routinely mowed, and access was restricted during periods of dry weather to ensure it was maintained. Upon introduction to the barn, 355 hens were placed into each of the four pens, and 110 randomly selected birds per pen were fitted with an RFID transponder (Hitag S 2,048 bits, 125 kHz) attached to an adjustable leg band (IDs, Roxan, Scotland). Artificial light was provided in the barn from 200 to 1,700 h, with transitional phases of five min beginning at 200 h and 15 min beginning at 1,645 h. Natural daylight was provided from 800 to 1,630 h through windows controlled by curtains. To allow hens to acclimatize to the barn interior, they were kept inside for the 1st week. Subsequent access to the wintergarden, stone yard, and range was first provided one, 2 and 4 weeks after population, respectively. For the subsequent 5-months period (June 7–October 16, 2016), birds were permitted weather-dependent voluntary daily access to the external areas. Antennae were positioned on either side of the transition points (popholes/gates) connecting two areas and RFID transponders recorded the date and time of each zone-transition made. Records permitted calculation of the time spent in each area [as in (4)], but not distances traveled within them. At the conclusion of the daily period for which birds were provided outside access, those in other areas were encouraged back into the barn interior.

At 42 weeks of age, a roughly equal number of tagged birds from each pen were haphazardly selected for sampling of hippocampal tissues and behavioral assessment (total $n = 58$). Loss of samples from five birds during molecular biology processing resulted in a final sample size of 53 birds.

## Ranging Quantification

Daily observations started at the time the range was opened and stopped when the range was closed. Based upon weather conditions, daily opening times varied between 7:50 and 13:50 and closing times ranged from 14:20–16:55, though hens were typically allowed to range until 16:30 each day. On days that behavioral testing, management protocols (vaccinations) or poor weather required restricted access to outdoor areas, access was intentionally restricted equally across all pens. The percentage of time spent in each area by each hen was calculated based on the remaining observation period of 72 days, wherein the range was accessible for an average of 6 h 58 min per day. There were some instances of loss of antenna signal coverage, generally caused by birds moving too quickly for detection. This meant that not all transitions were recorded. However, data checks confirmed accurate recording of each animal's movement and location patterns (e.g., sequential progression through areas rather than "jumps" to more distal ones). On average, the location of a bird was recorded for 83% of the time the range was open (mean coverage rate, IQR 69–96%). Proportions of available time spent in each area by hens were calculated based only upon times wherein their individual zonal locations were transmitted, leaving an average of 5 h 47 min tracked per day. As missed recordings were distributed evenly over all areas, while the actual times that birds spent outdoors/on the range may have been higher than observed, the proportional times analyzed should not have been affected. Detailed data regarding movement of hens between the barn and external areas is reported elsewhere (66).

## Tonic Immobility

As part of related experimental evaluations [reported elsewhere: (67)], collection of final measurements spanned a four-day period in which all hens were prevented from leaving the barn. On each day, hens from a single pen were transported from their home pen to another barn on-site for the measurement of TI, which occurred shortly before tissue collection. To induce immobility, hens were placed on their backs on a holding frame, with a light pressure applied to the breast. After pressure was released, the latency until the hen righted itself was timed using a stopwatch. The same observer (SGH) conducted all TI tests. If immobility was not successfully induced (i.e., the bird started to move within 3 s of removal of pressure), the procedure was repeated up to three times. Where immobility was not induced after the final attempt, the hen received a latency of 0 s. If a hen remained immobile for 300 s, they were ascribed this value as the maximum latency and the test was terminated.

## Tissue Collection

Shortly after TI measurement, animals ($n = 58$) were killed via intravenous injection with pentobarbital (Esconarkon, 0.3 ml/hen due to similar weights). Immediately thereafter, brains were removed from the skull, placed into 0.1 M phosphate-buffered saline in a Petri dish and divided along the longitudinal fissure with a scalpel. From each hemisphere, the HF was dissected and divided midway across the rostrocaudal axis to produce two subsamples (rostral and caudal) containing equal amounts of tissue. This method constitutes a rough estimate of the boundary between the rostral and caudal HF, as the exact border between these putative functional subdivisions has yet to be clearly mapped out. The 4 HF samples collected from each hen were processed separately. Isolated HF regions were placed in sample tubes containing 1.5 ml of RNAlater® Stabilization Solution (Thermo Fisher Scientific, UK) and refrigerated for 24 h before storage at −30°C.

## RNA Isolation and Reverse Transcription

RNA was extracted using TriSure reagent (Bioline, London, UK) and Lysing Matrix D tubes in a FastPrep Instrument (MP Biomedicals, Cambridge, UK). Purification of the RNA product combined with DNAse treatment was conducted with the Zymo Direct-zol™ RNA MiniPrep Kit (Cambridge Bioscience, Cambridge, UK), according to manufacturer's instructions. 2 μg RNA was reverse transcribed using the Tetro™ cDNA Synthesis Kit (Bioline, London, UK) for use in a quantitative real-time polymerase chain reaction (qPCR).

## Quantitative PCR

Gene specific primers were designed using the NCBI primer-BLAST tool and sequences are displayed in **Table 1**. As previously, the chicken lamin B receptor (LBR) gene was used as a control gene for normalization (68). Standards were produced by gel purification of PCR products using a MinElute gel extraction kit (Qiagen Ltd, Crawley, UK) and their concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Serial dilutions of standards were produced to create standard curves for qPCR quantification. qPCR reactions were run on a Bio-Rad model machine (Bio-Rad, California, USA). Reactions (20 μl) contained 5 μl of cDNA template together with 10 μl SYBR green master mix (No-ROX kit, Bioline, London, UK) and gene specific primers (400 nM). The manufacturer's instructions were followed for 3-step thermal cycling conditions. Samples were run in singlicate over three batches on a 96-well plate, each of which contained samples from animals across the spectrum of range use and was accompanied by a standard curve run in duplicate. No-template controls were also included. A melting curve analysis was performed to confirm specificity of reactions and efficiency values for the primers used ranged between 99.7 and 108.9%. Assays were analyzed using CFX-Manager software (Bio-Rad, California, USA).

## Statistical Analysis

Analyses were conducted in IBM SPSS Statistics (v24). Linear mixed models (LMMs) were conducted to explore how the proportional times spent in each of the four areas (barn, wintergarden, stone yard, and range) by hens were related, while accounting for experimental pen as a random factor. Times in the intermediate areas (wintergarden and stone yard) were included separately as covariates while times in the extreme

**TABLE 1 |** Gene-specific primers used for qPCR in tissue from the hippocampal formation.

| Gene | Accession | Orientation | Primer sequence (5′–3′) | Product length (bp) |
|---|---|---|---|---|
| LBR | NM_205342 | Forward | GGTGTGGGTTCCATTTGTCTACA | 80 |
| | | Reverse | CTGCAACCGGCCAAGAAA | |
| PCNA | NM_204170.2 | Forward | CAATGCGGATACGTTGGCTC | 192 |
| | | Reverse | ACAGCATCACCAATGTGGCT | |
| DCX | NM_204335.3 | Forward | AAGACGGCCCATTCGTTTGA | 166 |
| | | Reverse | ATTTTTCGGGACCACAGGCA | |

areas (barn and range) were dependent variables. Whether time in the wintergarden co-varied with time in the stone yard was also explored. Separate univariate ANOVAs were employed to determine whether TI durations and time spent in each area differed between experimental pens. A generalized linear model with a Poisson loglinear distribution was used to determine whether the number of attempts required to induce TI differed between pens. To explore whether ranging was related to TI durations, LMMs were conducted with TI duration as the dependent variable, pen as a random factor, number of attempts to induce TI as a fixed factor, and proportional time in each of the areas as covariates (over four individual models). Measured molar quantities of PCNA, DCX, and LBR mRNA were log(10)-transformed and, as the quantity of samples necessitated multiple qPCR runs, normalized using the Standard Score ($Z_i$) within assays. Separate LMMs with unstructured covariance were conducted for PCNA and DCX, each with HF subregion (rostral/caudal) and sample (one per hemisphere) as repeated fixed factors, pen as a random factor and LBR expression in the same sample as a covariate. In individual models, percentage of time spent in each area was included as a covariate, as well as in their interaction with HF subregion. Two models explored whether TI was related to expression of each gene and included TI duration as a covariate, TI attempts as a fixed factor, and both variables in an interaction term with HF subregion. Where both TI duration and time in an area co-varied significantly with expression of the same gene, they were also included together in a single LMM to verify their explanation of independent proportions of the variance. The corrected gene expression values plotted in **Figures 2**, **3** comprise residual PCNA and DCX after accounting for LBR expression, rostrocaudal subregion, sample and pen in LMMs, as described above.

# RESULTS

## Behavior

On average, the 53 focal hens spent the majority of available tracked time either in the barn or in the wintergarden (see **Table 2**). Proportional time in the wintergarden was therefore negatively correlated with time spent in the barn [$F_{(1,\ 50.9)} = 199.7$, $p < 0.001$, $B = -1.212$, SEM $= 0.086$]. One hen remained exclusively within the barn and never entered the wintergarden. Time spent in the wintergarden was positively correlated with time spent in the stone yard [$F_{(1,\ 50.5)} = 11.06$, $p = 0.002$, $B = 0.161$, SEM $= 0.049$], but did not predict time on the grassy range [$F_{(1,\ 50.5)} = 1.04$, $p = 0.313$, $B = 0.052$, SEM $= 0.051$]. Five hens never ventured outside (i.e., to the stone yard), while an additional three spent time in the stone yard but did not enter the grassy range. This meant that 45 hens (∼85%) thus used all areas provided to some extent. Time spent in the stone yard was positively correlated with time on the range [$F_{(1,\ 50.5)} = 18.12$, $p < 0.001$, $B = 0.494$, SEM $= 0.116$].

The mean number of daily transitions that hens made between areas correlated negatively with time in the barn [$F_{(1,\ 49.3)} = 118.75$, $p < 0.001$, $B = -0.9753$, SEM $= 0.089$] and positively with time in each of the three other areas [wintergarden: $F_{(1,\ 49.6)} = 49.95$, $p < 0.001$, $B = 0.5895$, SEM $= 0.087$; stone yard: $F_{(1,\ 49.3)} = 46.84$, $p < 0.001$, $B = 0.2236$, SEM $= 0.033$; range: $F_{(1,\ 49.5)} = 20.72$, $p < 0.001$, $B = 0.1670$, SEM $= 0.037$].

Behavior was compared between the four experimental pens. There was no difference in durations of TI between pens [$F_{(3,47)} = 1.10$, $p = 0.358$], and the number of attempts required to induce immobility did not differ ($\chi^2_3 = 3.86$, $p = 0.277$, see **Table 3**). Controlling for pen as a random factor, duration of the TI response did not differ with the number attempts to induce it [$F_{(2,44.9)} = 0.92$, $p = 0.406$].

In terms of ranging behavior, proportional time spent in the barn [$F_{(3,49)} = 1.52$, $p = 0.222$] and wintergarden [$F_{(3,49)} = 1.53$, $p = 0.219$] did not differ between pens. However, pens differed in the proportional time that hens spent in the stone yard [$F_{(3,49)} = 3.39$, $p = 0.025$, see **Figure 1**]. Hens in pen two ($M = 13.81$, SEM $= 3.81$) spent longer in the stone yard than hens in pen three ($M = 4.72$, SEM $= 1.74$; $p = 0.006$) and pen four ($M = 5.97$, SEM $= 1.44$; $p = 0.010$), with a trend toward longer times than pen one ($M = 8.81$, SEM $= 1.32$; $p = 0.088$). There was a trend toward differing proportional times spent on the range between pens [$F_{(3,49)} = 2.37$, $p = 0.082$]. Time spent on the range was higher for hens from pen one ($M = 9.53$, SEM $= 1.71$) than pens two ($M = 3.40$, SEM $= 2.22$; $p = 0.034$) and three ($M = 3.44$, SEM $= 2.12$; $p = 0.030$). Hens from pen four spent an intermediate amount of time on the range ($M = 5.80$, SEM $= 1.82$), which did not differ from the other three pens. Accounting for pen as a random factor and attempts needed to induce TI as a fixed factor, the duration of TI was not associated with time in any of the four areas [barn: $F_{(1,\ 46.8)} = 1.69$, $p = 0.200$; wintergarden: $F_{(1,\ 46.3)} = 0.58$, $p = 0.449$; stone yard: $F_{(1,\ 43.1)} = 2.94$, $p = 0.094$; range: $F_{(1,\ 46.2)} = 0.59$, $p = 0.448$].

## Hippocampal Gene Expression

As expected, expression of LBR mRNA covaried with expression of PCNA [$F_{(1,\ 88.5)} \geq 264.7$, $p < 0.001$] and DCX [$F_{(1,\ 75.8)} \geq$

**TABLE 2 |** Descriptive statistics for proportions of available time (%) spent in each of the four areas of the housing system by focal hens ($n = 53$) over the tracked period.

| | *N* | Mean | Standard deviation | Median | IQR | Range |
|---|---|---|---|---|---|---|
| Barn | 53 | 51.31 | 26.61 | 41.00 | 51.64 | 11.81–100.00 |
| Wintergarden | 52 | 34.54 | 19.61 | 40.47 | 35.22 | 0.00–66.07 |
| Stone yard | 48 | 8.10 | 7.70 | 7.16 | 11.37 | 0.00–33.72 |
| Range | 45 | 6.05 | 7.30 | 2.28 | 7.90 | 0.00–28.28 |

*n indicates the number of birds that spent at least some time in each area.*

**TABLE 3 |** Descriptive statistics for durations of tonic immobility and number of attempts required to induce the state for hens from the four experimental pens.

| | *N* | Median (s) | IQR (s) | Range (s) | *n* censored | Mean attempts |
|---|---|---|---|---|---|---|
| Pen 1 | 17 | 269.3 | 226.4 | 12.3–300.0 | 7 | 1.27 |
| Pen 2 | 10 | 290.0 | 155.4 | 60.1–300.0 | 5 | 2.30 |
| Pen 3 | 11 | 261.4 | 172.5 | 78.0–300.0 | 4 | 1.82 |
| Pen 4 | 15 | 109.7 | 282.8 | 6.5–300.0 | 3 | 1.93 |

*Censored times had the maximum duration of 300 s.*

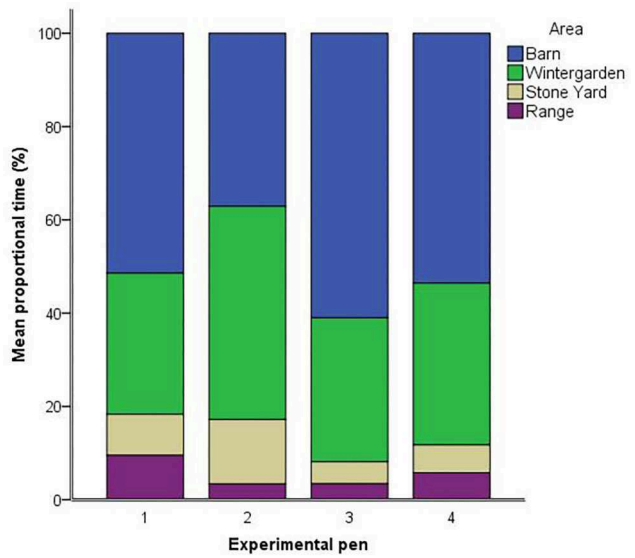


**FIGURE 1 |** Mean proportions of available tracked time (%) spent in each of the four areas by hens in the four experimental pens.

354.4, $p < 0.001$] over all models. Samples of the same HF subregion taken from the two hemispheres did not differ from each other in expression of either gene [PCNA: $F_{(1, 45.4)} \leq 0.296$, $p \geq 0.589$; DCX: $F_{(1, 47.5)} \leq 0.114$, $p \geq 0.738$]. Expression also did not differ between the rostral and caudal HF subregions for PCNA [$F_{(1, 62.0)} \leq 1.72$, $p \geq 0.194$] or DCX [$F_{(1, 51.3)} \leq 1.97$, $p \geq 0.166$] mRNA in any models.

## Ranging and Hippocampal Gene Expression

Proportional time spent in the barn did not correlate with PCNA mRNA expression across the whole HF [$F_{(1, 48.0)} = 1.59$, $p = 0.214$], though there was a trend toward an interaction with HF subregion [$F_{(1, 45.7)} = 3.38$, $p = 0.073$]. In the rostral HF, there was a trend toward a negative relationship between time in the barn and PCNA expression [$B = -0.0030$, SEM = 0.002, $F_{(1, 47.2)} = 3.17$, $p = 0.082$], with no relationship in the caudal HF [$B = 0.0005$, SEM = 0.001, $F_{(1, 40.2)} = 0.33$, $p = 0.570$]. PCNA expression was not associated with proportional time spent in the wintergarden [$F_{(1, 47.5)} = 0.03$, $p = 0.866$], and there was no interaction with subregion [$F_{(1, 46.1)} = 0.72$, $p = 0.401$].

Proportional time spent in the stone yard positively correlated with PCNA expression over the whole HF [$F_{(1, 46.6)} = 6.54$, $p = 0.014$, $B = 0.0018$, SEM = 0.003], and there was an interaction with subregion [$F_{(1, 44.7)} = 4.57$, $p = 0.038$]. While time in the stone yard positively correlated with PCNA mRNA in the rostral HF [$B = 0.0146$, SEM = 0.006, $F_{(1, 46.6)} = 6.46$, $p = 0.014$], there was no relationship in the caudal subregion [$B = 0.0010$, SEM = 0.033, $F_{(1, 34.4)} = 0.12$, $p = 0.729$].

The percentage of available time spent on the grassy range by hens did not correlate with their expression of PCNA mRNA in the HF as a whole [$F_{(1, 50.0)} = 2.95$, $p = 0.092$], but there was an interaction with rostrocaudal subregion [$F_{(1, 46.2)} = 5.10$, $p = 0.029$, **Figure 2A**]. Time on the range positively was positively associated with PCNA expression in the rostral HF [$B = 0.0123$, SEM = 0.006, $F_{(1, 45.6)} = 4.10$, $p = 0.049$] but not the caudal region [$B = -0.0021$, SEM = 0.003, $F_{(1, 41.8)} = 0.46$, $p = 0.501$]. As time in the stone yard and time on the grassy range correlated positively with each other and both related to PCNA expression, an association between their combined values (i.e., the total available time spent outdoors) and PCNA levels was also explored. Time outdoors was related to PCNA expression [$F_{(1, 50.6)} = 6.75$, $p = 0.012$, $B = 0.0004$, SEM = 0.002] and an interaction [$F_{(1, 44.7)} = 6.64$, $p = 0.013$] indicated that this association was again attributable to a positive relationship in the rostral HF [$F_{(1, 46.0)} = 7.36$, $p = 0.009$, $B = 0.0092$, SEM = 0.003], with no correlation in the caudal subregion [$F_{(1, 39.0)} = 0.02$, $p = 0.895$, $B = -0.0002$, SEM = 0.002].

Similarly, time spent on the range did not co-vary with hippocampal DCX expression over the whole HF

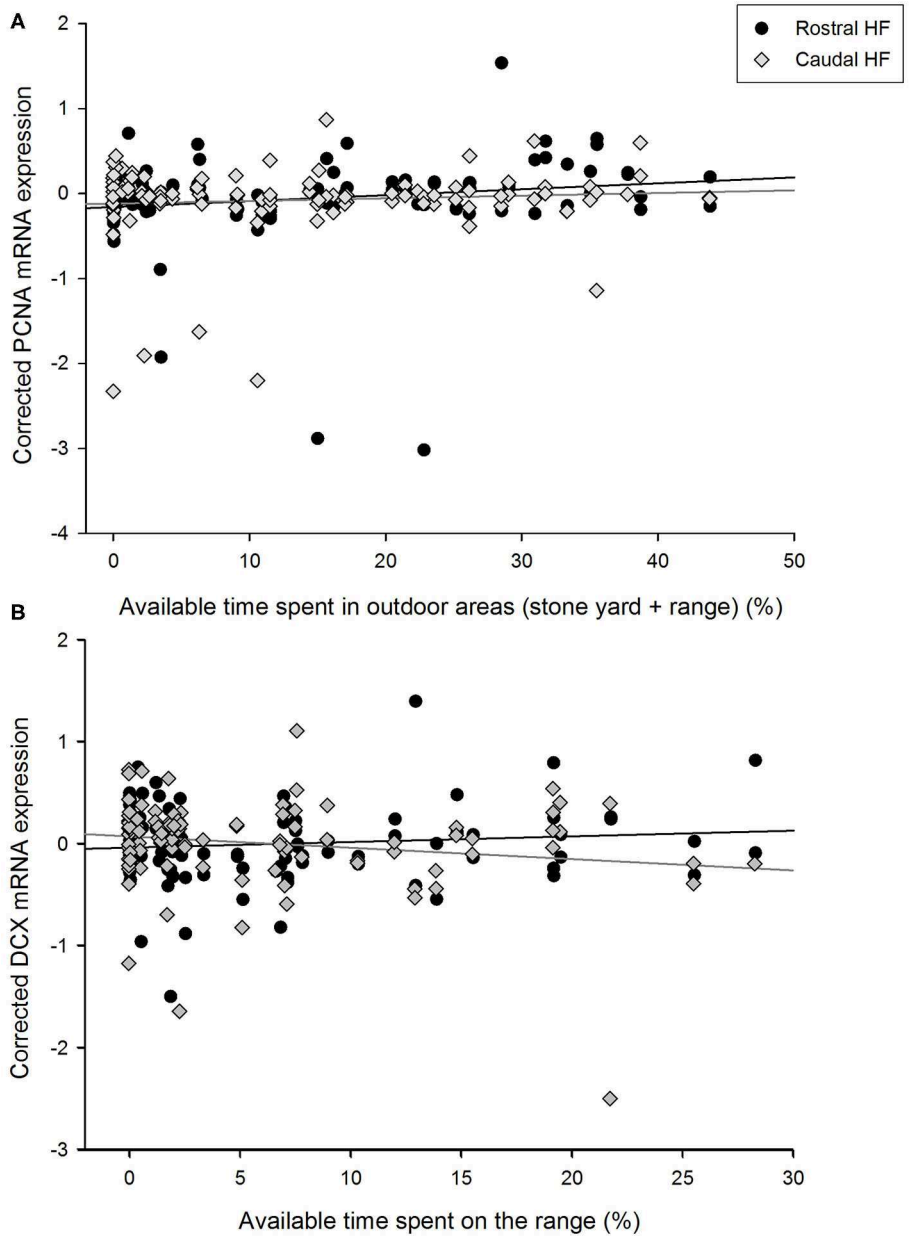


**FIGURE 2 |** Relationships between the proportions of available time spent in outdoor areas by individual hens and corrected gene expression in the rostral and caudal subregions of the hippocampal formation. **(A)** PCNA expression in relation to the total percentage of time spent in outdoor areas (i.e., the stone yard + grassy range) by focal hens. **(B)** Doublecortin (DCX) expression in relation to the percentage of time that focal hens spent on the grassy range. Gene expression values are unstandardized residuals following correction for LBR expression, rostrocaudal subregion, sample, and pen, in linear mixed models.

[$F_{(1,\ 51.4)} = 0.56$, $p = 0.456$] but the difference in slopes between the rostral and caudal subregions was significant [$F_{(1,\ 54.8)} = 4.72$, $p = 0.034$, **Figure 2B**]. Time spent on the range was not associated with DCX expression in the rostral HF [$B = 0.0062$, SEM $= 0.070$, $F_{(1,\ 49.1)} = 1.12$, $p = 0.296$] but negatively correlated with DCX expression in the caudal HF [$B = -0.0140$, SEM $= 0.007$, $F_{(1,\ 53.2)} = 4.09$, $p = 0.048$]. DCX expression was not associated with time in any other area [barn: $F_{(1,\ 49.1)} = 0.02$, $p = 0.896$; wintergarden: $F_{(1,\ 49.2)} = 0.11$, $p = 0.744$; stone yard: $F_{(1,\ 50.3)} = 1.25$, $p = 0.270$], nor did these parameters interact with HF subregion [time in barn*subregion: $F_{(1,\ 51.0)} = 0.17$, $p = 0.679$; time in wintergarden*subregion: $F_{(1,\ 51.1)} = 0.26$, $p = 0.613$; time in stone yard*subregion: $F_{(1,\ 50.9)} = 0.96$, $p = 0.332$].

Lastly, the mean number of daily transitions between the four areas made by individual hens (a crude proxy for activity levels) did not correlate with expression of hippocampal PCNA [$F_{(1,\ 47.1)} = 1.25$, $p = 0.261$], though there was a trend toward

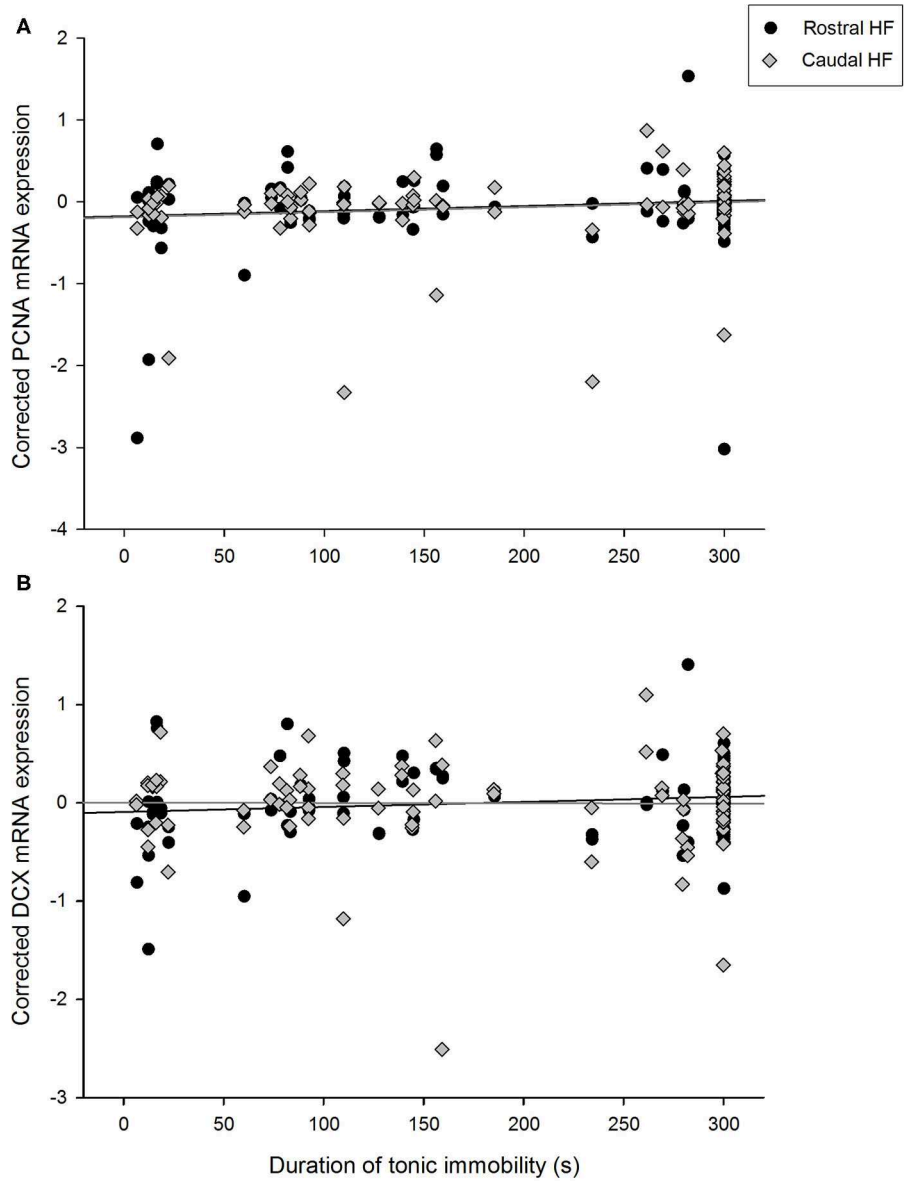


**FIGURE 3 |** Relationship between durations of tonic immobility (seconds) for individual hens and residual expression of **(A)** PCNA and **(B)** doublecortin (DCX) in rostral and caudal subregions of the hippocampal formation, after correcting for LBR expression, rostrocaudal subregion, sample, and pen in linear mixed models.

an interaction with subregion [$F_{(1,\ 44.7)} = 3.22$, $p = 0.080$], with the slopes of the relationships tending in opposite directions [rostral: $B = 0.0025$, SEM $= 0.002$, $F_{(1,\ 45.7)} = 1.64$, $p = 0.206$; caudal: $B = -0.0007$, SEM $= 0.001$, $F_{(1,\ 41.0)} = 0.60$, $p = 0.443$]. DCX expression was not associated with number of transitions [$F_{(1,\ 49.0)} = 0.01$, $p = 0.915$; transitions*subregion $F_{(1,\ 51.0)} = 0.03$, $p = 0.855$].

## Tonic Immobility and Hippocampal Gene Expression

Duration of TI positively correlated with expression of PCNA over the whole HF [$F_{(1,\ 45.0)} = 5.60$, $p = 0.022$] and did not interact with rostrocaudal subregion [$F_{(1,\ 40.9)} = 0.43$, $p = 0.516$, **Figure 3A**]. PCNA expression did not differ between hens requiring one, two or three attempts to induce TI [$F_{(2,39.8)} = 1.46$, $p = 0.245$], nor did number of attempts interact with subregion [$F_{(2,40.5)} = 0.83$, $p = 0.443$]. Conversely, hippocampal DCX mRNA expression was not associated with TI duration [$F_{(1,\ 47.4)} = 0.68$, $p = 0.412$] and there was no interaction with rostrocaudal subregion [$F_{(1,\ 49.2)} = 0.96$, $p = 0.092$, $p = 0.333$, **Figure 3B**]. DCX expression did not differ with attempts to induce TI [$F_{(2,46.6)} = 0.97$, $p = 0.386$], and there was no interaction with subregion [$F_{(2,46.6)} = 1.47$, $p = 0.241$].

To verify that proportions of time spent outdoors and durations of TI explained independent proportions of the variance in PCNA expression, they were included as covariates

in the same model. TI duration continued to co-vary with PCNA expression throughout the HF [$F_{(1, 43.2)} = 4.39$, $p = 0.042$], and PCNA expression did not differ with the number of induction attempts [$F_{(2,39.5)} = 1.34$, $p = 0.273$]. Proportional time in outdoor areas co-varied with overall PCNA expression [$F_{(1, 47.0)} = 5.72$, $p = 0.021$], and the interaction between time outdoors and HF subregion remained significant [$F_{(1, 42.1)} = 6.01$, $p = 0.018$].

# DISCUSSION

This study constituted an early exploration of the associations between individual differences and hippocampal plasticity in domestic chickens. Within the sampled flock of laying hens, individual differences in durations of TI were not correlated with time spent in any area of the housing system. Differences in TI have previously been reported between ranging sub-groups (69, 70), but another RFID-tracking study also failed to observe a relationship at the individual level (71). In the present sample, TI and use of the outdoor areas each explained separate portions of the total variance in expression of proliferative marker PCNA. The findings therefore support the existence of two independent relationships that link these behaviors to proliferation in the HF, each partially consistent with the hypothesized mechanisms.

Durations of TI positively correlated with expression of proliferative marker PCNA over the whole HF, suggesting that fearful, reactive hens have a higher level of hippocampal proliferation. This association is consistent with the predicted relationship between AHN and coping style. However, reactive hens would be expected to be less exploratory, but the associations between proliferation in the rostral HF subregion and proportional time spent in the furthest areas from the barn (the outdoor stone yard and range) were also positive. These subregional relationships are therefore more consistent with the predicted stimulatory effect of ranging experience on hippocampal cell proliferation. DCX expression, indicative of neuronal differentiation, displayed a generally different pattern from AHN cell proliferation: it negatively co-varied with proportion of time spent on the range, but only in the caudal HF. We will discuss each of the findings separately below.

## Hippocampal Gene Expression and Tonic Immobility (Coping Style)

Both higher basal levels of hippocampal proliferation and longer durations of TI are traits characteristic of individuals exhibiting a reactive (or passive) behavioral strategy/coping style (36, 37). Individual differences in proliferation, but not survival, have been positively related to the degree of freezing vs. locomotion displayed by rats in a novel environment (37). Supporting a causal contribution of new cells to reactivity, mice with experimentally-suppressed neurogenesis freeze less than wild-type mice when faced with a novel environment and stimulus during contextual fear conditioning (72). Reactive individuals also display enhanced behavioral flexibility (22), and hippocampal neurogenesis is necessary for flexible behavior during learning tasks (39, 40). It is theorized that adult-born neurons promote the erasure of previously learned associations, in order to minimize proactive interference and facilitate the acquisition of novel associations (73). Adult-born neurons have also been demonstrated to inhibit the activity of mature granule cells under conditions of novelty and anxiety (74, 75). As such, AHN may form part of the intrinsic mechanism which links individual differences in cognitive flexibility to those in behavioral responses to challenge. A higher level of proliferation may translate to a relatively higher number of surviving new neurons under certain conditions (76), but current research does not indicate how proliferating cells may exert functional effects prior to maturation and integration. A corresponding relationship between TI and expression of DCX would therefore be expected, and the absence of such a correlation may relate to the influence of environmental factors on neuronal differentiation, or to a methodological explanation, each discussed below. Furthermore, though reactive individuals are often less exploratory (16, 17), no relationship between freezing (TI) and ranging existed for the sampled flock. Previous studies in hens have explicitly linked behavioral flexibility, but not fearfulness, to ranging tendencies (26, 27). It may be that other dimensions of personality, such as sociability (77), are also influential determinants of ranging behavior, and obscure a simple relationship with reactivity.

## Ranging Experience and PCNA Expression

We also observed a significant positive relationship between ranging outside and PCNA expression, and this was specific to the rostral HF. Based on neuroanatomy, the dorsal rodent and rostral avian regions are hypothesized to be homologous, while the caudal avian HF is hypothesized to be homologous to the ventral rodent hippocampus (63). As TI durations were not associated with ranging in terms of the relative time spent in any area (internal or external), the relationship between ranging and PCNA expression is unlikely to relate to coping style. Instead, it may reflect the influence of ranging experience on hippocampal plasticity. In the rostral HF, time spent in both outdoor areas (the stone yard and grassy range) was positively associated with expression of *PCNA*. This relationship may be attributable to the stimulatory effect of factors including environmental complexity and exercise on hippocampal proliferation, as such experiences have been observed to preferentially modulate AHN in the dorsal rodent HF (48, 50, 78).

While the multi-tier barn interior comprises a complex, three-dimensional environment, all hens necessarily spent a substantial proportion of their time there: during the night and at other times that the additional areas were closed. Moreover, individual hens remained within the barn for a minimum of ∼12% of the time that all areas were open, and it was the only location wherein certain key resources, including feed and nest boxes, were provided. Therefore, while the barn interior likely already comprised a cognitively challenging environment that could be considered enriched, this experience was shared by all birds. The wintergarden also provided resources in the form of drinkers and perches, and perhaps represented an extension of the barn

in that it was used by all but one hen. In contrast, the lower proportion of hens that also regularly ventured farther afield, into the uncovered stone yard and range, effectively had a larger home range. This may entail maintenance of a larger mental map, while presenting greater navigational challenge to return to the resources provided inside. Size of the home range positively predicts hippocampal plasticity across species of rodent [reviewed in (79)], and the variety of territory coverage by individual mice roaming a complex home environment was strongly correlated with AHN (28). In birds, AHN rates are higher in migratory than non-migratory subspecies (of white crowned sparrow, *Zonotrichia leucophrys*) (80) and are stimulated by spatial-cognitive demand in experimental settings (57, 81), An increase in HF proliferation was observed following the storage and retrieval of food caches by Marsh tits (*Poecile palustris*) (81), making the spatial-cognitive challenge of ranging outside a likely contributor to the observed relationship with PCNA expression. While environmental enrichment has been found to upregulate numbers of proliferating cells in mice (78, 82–84), physical activity is perhaps a more robust driver of expansion of the precursor cell pool (42, 76). There was a trend for the number of transitions that hens made between areas to predict rostral PCNA expression, which may also point to a similar relationship between exercise and proliferation in chickens. However, as it was not possible to measure the individual distances traveled within each area, this measure provides only a crude proxy, and future experimental work will be needed to establish such an association.

## Ranging Experience and DCX Expression

Given that the stimulation of proliferation by a positive experience such as exercise leads to a subsequent increase in the number of surviving new-born neurons (76), the lack of corresponding positive relationships between time in the outdoor areas and rostral expression of *DCX* is also surprising. This finding further conflicts with the robust effect of enrichment on later stages of AHN (41, 42). Moreover, in the caudal HF, time spent on the grassy range alone correlated negatively with *DCX* expression. Downregulation of AHN consistently occurs following the experience of stress, and the ventral hippocampus in rodents (85, 86) and the caudal HF in laying hens (61) are known to be particularly sensitive. As certain forms of stress have a greater negative influence on the later survival of young neurons (87–89), it is possible that this factor is responsible for the decoupling of relationships with PCNA and DCX expression. The observed negative relationship between time on the range and caudal DCX expression implies that, while outdoor visits provide further environmental complexity (and possibly exercise), hens that spend more time on the range also experience more stress. This association is perhaps related to the consistent finding that many hens provided with outdoor access choose not to range (4–7). Indeed, the general assumption that the range represents an exclusively positive environment has not been demonstrated.

There is some evidence to suggest that enrichment may be stressful even within controlled laboratory settings. One study found that enriched housing including running wheels upregulated DCX immunoreactivity and mRNA expression in the dorsal mouse dentate gyrus, while suppressing levels in the ventral region (49). Housing domestic pigeons in an enriched environment has also been observed to increase the number of DCX-expressing neurons in the HF (rostral and caudal HF were not distinguished), while simultaneously increasing average durations of TI (56). Though a group effect was observed, the authors found no correlation between TI times and cell numbers on an individual level. Further investigation into whether some general aspects of enrichment, perhaps relating to the cognitive challenge, are intrinsically associated with stress may therefore be warranted.

Beyond laboratory enrichment, ranging outdoors may expose hens to unpredictable sources of stress, such as adverse weather conditions and sightings of predators. Individual range use was previously positively correlated with the CORT response to handling and flightiness to avoid a human (71), which may indicate greater anxiety. As time in the stone yard was not negatively associated with DCX expression, the characteristics which distinguish the range itself may be particularly stressful. Both areas were uncovered, potentially exposing hens to rain and sightings of aerial predators, but weather conditions such as wind may be more salient on the large, open range than in the smaller, fenced stone yard. Contact with soil is also associated with exposure to a greater burden of parasites (90), which may be a source of immune-stress. Perhaps due to extensive cover in the ancestral environment of red junglefowl, hens show a collective preference for shelter (91), whereas use of the open range entails being exposed. Over multiple commercial farms, the number of birds using an outside range correlates positively with the amount of tree cover provided (92), while addition of tree cover or shelters increases use of the range (93). The range in the present study was relatively barren and did not contain trees or other forms of shelter. Consistent with the sampled hens spending less time on the range than in other areas, nearest-neighbor-distance is generally observed to increase with increasing distance from the barn (2). This lack of proximity to conspecifics may be stressful, due to greater perceived predation risk or social isolation. On the other hand, as frequently ranging hens choose to be less close to their conspecifics (77), it is also possible that hens visit the range to escape social conflict with flock mates. In this case, the experience of stress would drive visits outside. However, we would therefore also expect to see an association with coping style, meaning this explanation is probably not consistent with the lack of correlation between ranging and TI.

In mice, neuronal survival to the point of maturation may be promoted specifically within the dorsal hippocampus by environmental enrichment (78, 84). Recent research in laying hens indicates that neuronal differentiation may be suppressed preferentially at the caudal pole (61) or over the whole HF (62) by different sources of chronic stress. If spending time outside is indeed a stressful experience for hens, then the stimulatory influence of cognitive stimulation may have counteracted the suppressive effect of stress in the rostral subregion, leaving an observable negative relationship only at the caudal pole. In mice, a combination of experimental stress and cognitive stimulation in the form of maze learning

led to a preferential reduction in AHN in the ventral subregion (51). Such an interaction could explain the lack of a positive relationship between time spent outside and (rostral) DCX expression. In the case of proliferation, some studies have also found environmental enrichment to elevate levels specifically within the dorsal mouse hippocampus (84), while others have noted an increase in both subregions (78). Proliferating cell numbers may be reduced most severely in the ventral subregion by chronic stress (85), or be suppressed uniformly (84). It is therefore difficult to conclude whether an influence of stress on proliferation in the caudal HF may have contributed to the rostral-specific nature of the association between outdoor ranging and PCNA expression in the present study.

### Methodological Considerations

It is important to note that, while transcription of the DCX gene has been demonstrated to be a valid proxy for the effects of running on neuronal differentiation in mice (49), this association has yet to be verified in birds. Unlike in the mammalian brain, adult neurogenesis is not restricted to a single subdivision of the avian HF (equivalent to the dentate gyrus), meaning it is not possible to micro-dissect a particular substructure or to use a control gene specific to its cellular population (as with Prox1 for granule cells) for normalization. Previous research has noted background expression of DCX mRNA in non-neurogenic subdivisions of the mouse hippocampus, with levels unresponsive to running exercise (94). Though the majority of the avian telencephalon is neurogenic, low-level transcription of DCX in other types of HF cell, such as mature neurons undergoing dendrite-remodeling (95), might obscure correlations with expression of the marker by differentiating immature neurons. This issue of background expression relates specifically to the use of DCX as a marker, meaning our results for PCNA expression may be more reliable. Overall, while our findings suggest interesting relationships between behavior and AHN in domestic chickens, they would need to be validated using standard morphological techniques to quantify neurogenesis. The small effect sizes observed may reflect a complex interaction between the multiple internal and external factors which relate to AHN, but could also be linked to post-transcriptional processes which complicate the relationship between mRNA and protein levels (96). The specificity of such effects to the HF must also be confirmed by quantifying AHN in a control region of the telencephalon. Given that we are still working to establish the precise boundaries between the rostral/caudal subregions in our wider research, and this work therefore constitutes an early dataset that is building toward a better understanding of this hippocampal subdivision in birds.

## CONCLUSION

To conclude, individual differences in time spent on the free range and durations of TI in a commercial laying hen flock both positively correlated with cell proliferation in the HF (time on the range only in the rostral half), but were not related to each other. As found in other species, reactive hens had higher basal PCNA expression in the hippocampus, while exercise and enrichment through ranging were positively associated with PCNA expression in the rostral HF. Hippocampal proliferation thus most likely reflects both personality in terms of behavioral strategy/coping style and the influence of experience. On the other hand, expression of neuronal differentiation marker DCX in the caudal HF is negatively related to ranging experience. As the caudal HF may be preferentially sensitive to stress, it is thus possible that some aspects of ranging are both stimulating and stressful at the same time. However, this effect needs to be confirmed. Overall, individual differences in behavior are reflected in hippocampal plasticity, but probably for a number of different reasons.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

Experimental use of the animals was approved by the Bern Kantonal Authority (BE-46/16) and the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549).

## AUTHOR CONTRIBUTIONS

EA collected tissue, conducted the molecular laboratory work, and drafted the manuscript. MT conceived of and designed the ranging study. BV, SV, and SG-H analyzed the ranging data. TS, TB, JG, and VS coordinated the AHN study. All authors gave final approval for publication.

## FUNDING

Work was funded by the Eva Husi Stiftung and Haldimann Stiftung (both Switzerland) and the Universities Federation for Animal Welfare (UFAW).

## ACKNOWLEDGMENTS

We also thank Markus Schwab for providing excellent technical support and the staff of the Aviforum for caring for the animals.

## REFERENCES

1. Knierim U. Animal welfare aspects of outdoor runs for laying hens: a review. *NJAS.* (2006) 54:133–45. doi: 10.1016/S1573-5214(06)80017-5
2. Chielo LI, Pike T, Cooper J. Ranging behaviour of commercial free-range laying hens. *Animals.* (2016) 6:28. doi: 10.3390/ani6050028
3. Campbell DLM, Talk AC, Loh ZA, Dyall TR, Lee C. Spatial cognition and range use in free-range laying hens. *Animals.* (2018) 8:26. doi: 10.3390/ani8020026

4. Gebhardt-Henrich SG, Toscano MJ, Fröhlich EKF. Use of outdoor ranges by laying hens in different sized flocks. *Appl Anim Behav Sci.* (2014) 155:74–81. doi: 10.1016/j.applanim.2014.03.010
5. Richards GJ, Wilkins LJ, Knowles TG, Booth F, Toscano MJ, Nicol CJ, et al. Continuous monitoring of pop hole usage by commercially housed free-range hens throughout the production cycle. *Vet Rec.* (2011) 169:338. doi: 10.1136/vr.d4603
6. Campbell DLM, Hinch GN, Dyall TR, Warin L, Little BA, Lee C. Outdoor stocking density in free-range laying hens: radio-frequency identification of impacts of range use. *Animal*. (2017) 11:121–30. doi: 10.1017/S1751731116001154
7. Larsen H, Cronin GM, Gebhardt-Henrich SG, Smith CL, Hemsworth PH, Rault JL. Individual ranging behaviour patterns in commercial free-range layers as observed through RFID tracking. *Animals*. (2017) 7:21. doi: 10.3390/ani7030021
8. Roche DG, Careau V, Binning SA. Demystifying animal "personality" (or not): why individual variation matters to experimental biologists. *J Exp Biol.* (2016) 219:3832–43. doi: 10.1242/jeb.146712
9. Erhard HW, Mendl M. Tonic immobility and emergence time in pigs—more evidence for behavioural strategies. *Appl Anim Behav Sci.* (1999) 61:227–37. doi: 10.1016/S0168-1591(98)00196-8
10. Benus RF, Bohus B, Koolhaas JM, Van Oortmerssen GA. Heritable variation for aggression as a reflection of individual coping strategies. *Experientia.* (1991) 47:1008–19. doi: 10.1007/BF01923336
11. Erhard HW, Mendl M. Tonic immobility in pigs: two interpretations—coping strategies or fear. In: Forbes JM, Lawrence TLJ,Rodway RG, Varley MA, editors. *Animal Choices.* Edinburgh: British Society of Animal Science (1997). p. 109–10. doi: 10.1017/S0263967X00043548
12. Edelaar P, Serrano D, Carrete M, Blas J, Potti J, Tella JL. Tonic immobility is a measure of boldness towards predators: an application of Bayesian structural equation modeling. *Behav Ecol.* (2012) 23:619–26. doi: 10.1093/beheco/ars006
13. Broom DM, Knowles TG. The Assessment of Welfare During the Handling and Transport of Spent Hens. In: Faure JM, editor. *3rd European Symposium on Poultry Welfare*. Tours: World Poultry Science Association (1989).
14. Gallup GGJ, Ledbetter DH, Maser JD. Strain differences among chickens in tonic immobility: evidence for an emotionality component. *J Comp Physiol Psychol.* (1976) 90:1075–81. doi: 10.1037/h0078662
15. Nakayama S, Nishi Y, Miyatake T. Genetic correlation between behavioural traits in relation to death-feigning behaviour. *Popul Ecol.* (2010) 52:329–35. doi: 10.1007/s10144-009-0188-7
16. Benus RF, Den Daas S, Koolhaas JM, Van Oortmerssen GA. Routine formation and flexibility in social and non-social behaviour of aggressive and non-aggressive male mice. *Behaviour.* (1990) 112:185. doi: 10.1163/156853990X00185
17. Hall ML, Van Asten T, Katsis AC, Dingemanse NJ, Magrath MJL, Mulder R, et al. Animal personality and pace-of-life syndromes: do fast-exploring fairy-wrens die young? *Front Ecol Evol.* (2015) 3:28. doi: 10.3389/fevo.2015.00028
18. Sih A, Del Giudice M. Linking behavioural syndromes and cognition: a behavioural ecology perspective. *Proc Royal Soc B.* (2012) 367:216. doi: 10.1098/rstb.2012.0216
19. Zidar J, Balogh A, Favati A, Jensen P, Leimar O, Sorato E, et al. The relationship between learning speed and personality is age- and task-dependent in red junglefowl. *Behav Ecol Sociobiol.* (2018) 72:168. doi: 10.1007/s00265-018-2579-2
20. Guillette LM, Reddon AR, Hurd PL, Sturdy CB. Exploration of a novel space is associated with individual differences in learning speed in black-capped chickadees, *Poecile atricapillus*. *Behav Processes.* (2009) 82:265–70. doi: 10.1016/j.beproc.2009.07.005
21. Coppens CM, De Boer SF, Koolhaas JM. Coping styles and behavioural flexibility: towards underlying mechanisms. *Philos Trans Royal Soc B.* (2010) 365:217. doi: 10.1098/rstb.2010.0217
22. Höglund E, Silva PIM, Øverli O. Contrasting coping styles meet the wall: a dopamine driven dichotomy in behavior and cognition. *Front Neurosci.* (2017) 11:383. doi: 10.3389/fnins.2017.00383
23. Mcgraw CPKWR. Genetic differences in susceptibility of rats to the immobility reflex ("animal hypnosis"). *Behav Genet.* (1973) 3:155–61. doi: 10.1007/BF01067655
24. Guillette LM, Reddon AR, Hoeschele M, Sturdy CB. Sometimes slower is better: slow-exploring birds are more sensitive to changes in a vocal discrimination task. *Proc Royal Soc B.* (2010) 278:1669. doi: 10.1098/rspb.2010.1669
25. Zidar J, Balogh ACV, Leimar O, Løvlie H. Generalization of learned preferences covaries with behavioral flexibility in red junglefowl chicks. *Behav Ecol.* (2019) 30:1375–81. doi: 10.1093/beheco/arz088
26. Ferreira VHB, Peuteman B, Lormant F, Valenchon M, Germain K, Brachet M, et al. Relationship between ranging behavior and spatial memory of free-range chickens. *Behav Processes.* (2019) 166:103888. doi: 10.1016/j.beproc.2019.103888
27. Ferreira VHB, Reiter L, Germain K, Calandreau L, Guesdon V. Uninhibited chickens: ranging behaviour impacts motor self-regulation in free-range broiler chickens (*Gallus gallus domesticus*). *Biol Lett.* (2020) 16:721. doi: 10.1098/rsbl.2019.0721
28. Freund J, Brandmaier AM, Lewejohann L, Kirste I, Kritzler M, Krüger A, et al. Emergence of individuality in genetically identical mice. *Science.* (2013) 340:756–9. doi: 10.1126/science.1235294
29. Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comput Neurol.* (1965) 124:319–35. doi: 10.1002/cne.901240303
30. Cameron HA, Woolley CS, Mcewen BS, Gould E. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience.* (1993) 56:337–44. doi: 10.1016/0306-4522(93)90335-D
31. Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. *Nat Med.* (1998) 4:1313–7. doi: 10.1038/3305
32. Van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. *Nature.* (2002) 415:1030–4. doi: 10.1038/4151030a
33. Kempermann G, Jessberger S, Steiner B, Kronenberg G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* (2004) 27:447–52. doi: 10.1016/j.tins.2004.05.013
34. Prickaerts J, Koopmans G, Blokland A, Scheepens A. Learning and adult neurogenesis: survival with or without proliferation? *Neurobiol Learn Mem.* (2004) 81:1–11. doi: 10.1016/j.nlm.2003.09.001
35. Zhang J, Jiao J. Molecular biomarkers for embryonic and adult neural stem cell and neurogenesis. *Biomed Res Int.* (2015) 2015:727542. doi: 10.1155/2015/727542
36. Vindas MA, Gorissen M, Höglund E, Flik G, Tronci V, Damsgård B, et al. How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. *J Exp Biol.* (2017) 220:1524–32. doi: 10.1242/jeb.153213
37. Lemaire V, Aurousseau C, Le Moal M, Abrous DN. Behavioural trait of reactivity to novelty is related to hippocampal neurogenesis. *Eur J Neurosci.* (2008) 11:4006–14. doi: 10.1046/j.1460-9568.1999.00833.x
38. Øverli Ø, Sørensen C. On the role of neurogenesis and neural plasticity in the evolution of animal personalities and stress coping styles. *Brain Behav Evol.* (2016) 87:167–74. doi: 10.1159/000447085
39. Garthe A, Behr J, Kempermann G. Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. *PLoS ONE.* (2009) 4:e5464. doi: 10.1371/journal.pone.0005464
40. Burghardt NS, Park EH, Hen R, Fenton AA. Adult-born hippocampal neurons promote cognitive flexibility in mice. *Hippocampus.* (2012) 22:1795–808. doi: 10.1002/hipo.22013
41. Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. *Nature.* (1997) 386:493–5. doi: 10.1038/386493a0
42. Van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci.* (1999) 2:266–70. doi: 10.1038/6368
43. Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J. Neurosci.* (2000) 20:9104–10. doi: 10.1523/JNEUROSCI.20-24-09104.2000
44. Manev H, Uz T, Smalheiser NR, Manev R. Antidepressants alter cell proliferation in the adult brain in vivo and in neural cultures in vitro. *Eur J Pharmacol.* (2001) 411:67–70. doi: 10.1016/s0014-2999(00)00904-3

45. Gould E, Tanapat P, Mcewen BS, Flügge G, Fuchs E. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc Natl Acad Sci USA*. (1998) 95:3168–71. doi: 10.1073/pnas.95.6.3168
46. O'leary OF, Cryan JF. A ventral view on antidepressant action: roles for adult hippocampal neurogenesis along the dorsoventral axis. *Trends Pharmacol Sci.* (2014) 35:675–87. doi: 10.1016/j.tips.2014.09.011
47. Fanselow MS, Dong H. Are The Dorsal and Ventral Hippocampus functionally distinct structures? *Neuron.* (2010) 65:7. doi: 10.1016/j.neuron.2009.11.031
48. Ramirez-Rodriguez G, Ocana-Fernandez MA, Vega-Rivera NM, Torres-Perez OM, Gomez-Sanchez A, Estrada-Camarena E, et al. Environmental enrichment induces neuroplastic changes in middle age female Balb/c mice and increases the hippocampal levels of BDNF, p-Akt and p-MAPK1/2. *Neuroscience.* (2014) 268:158–70. doi: 10.1016/j.neuroscience.2013.12.026
49. Gualtieri F, Brégère C, Laws GC, Armstrong EA, Wylie NJ, Moxham TT, et al. Effects of environmental enrichment on doublecortin and BDNF expression along the dorso-ventral axis of the dentate gyrus. *Front Neurosci.* (2017) 11:488. doi: 10.3389/fnins.2017.00488
50. Nishijima T, Kawakami M, Kita I. Long-term exercise is a potent trigger for ΔFosB induction in the hippocampus along the dorso–ventral axis. *PLoS ONE.* (2013) 8:e81245. doi: 10.1371/journal.pone.0081245
51. Hawley DF, Morch K, Christie BR, Leasure JL. Differential response of hippocampal subregions to stress and learning. *PLoS ONE.* (2012) 7:e53126. doi: 10.1371/journal.pone.0053126
52. Schloesser RJ, Lehmann M, Martinowich K, Manji HK, Herkenham M. Environmental enrichment requires adult neurogenesis to facilitate the recovery from psychosocial stress. *Mol Psychiatry.* (2010) 15:1152–63. doi: 10.1038/mp.2010.34
53. Bouillé C, Baylé JD. Effects of limbic stimulations or lesions on basal and stress induced hypothalamic pituitary adrenocortical activity in the pigeon. *Neuroendocrinology.* (1973) 13:264–77. doi: 10.1159/000122211
54. Colombo M, Broadbent N. Is the avian hippocampus a functional homologue of the mammalian hippocampus? *Neurosci Biobehav Rev.* (2000) 24:465–84. doi: 10.1016/S0149-7634(00)00016-6
55. Striedter GF. Evolution of the hippocampus in reptiles and birds. *J Comp Neurol.* (2016) 524:496–517. doi: 10.1002/cne.23803
56. Melleu FF, Pinheiro MV, Lino-De-Oliveira C, Marino-Neto J. Defensive behaviours and prosencephalic neurogenesis in pigeons (*Columba livia*) are affected by environmental enrichment in adulthood. *Brain Struct Funct.* (2016) 221:2287–301. doi: 10.1007/s00429-015-1043-6
57. Ladage LD, Roth TC, Fox RA, Pravosudov VV. Ecologically-relevant spatial memory use modulates hippocampal neurogenesis. *Proc Royal Soc B.* (2010) 277:1071–9. doi: 10.1098/rspb.2009.1769
58. Barnea A, Pravosudov VV. Birds as a model to study adult neurogenesis: bridging evolutionary, comparative and neuroethological approaches. *Eur J Neurosci.* (2011) 34:884–907. doi: 10.1111/j.1460-9568.2011.07851.x
59. Robertson B, Rathbone L, Cirillo G, D'eath RB, Bateson M, Boswell T, et al. Food restriction reduces neurogenesis in the avian hippocampal formation. *PLoS ONE.* (2017) 12:e0189158. doi: 10.1371/journal.pone.0189158
60. Taufique SKT, Prabhat A, Kumar V. Constant light environment suppresses maturation and reduces complexity of new born neuron processes in the hippocampus and caudal nidopallium of a diurnal corvid: implication for impairment of the learning and cognitive performance. *Neurobiol Learn Mem.* (2018) 147:120–7. doi: 10.1016/j.nlm.2017.12.001
61. Gualtieri F, Armstrong E, Longmoor G, D'eath RB, Sandilands V, Boswell T, et al. Unpredictable chronic mild stress suppresses the incorporation of new neurons at the caudal pole of the chicken hippocampal formation. *Sci Rep.* (2019) 9:7129. doi: 10.1038/s41598-019-43584-x
62. Armstrong EA, Rufener C, Toscano MJ, Eastham JE, Guy JH, Sandilands V, et al. Keel bone fractures induce a depressive-like state in laying hens. *Sci Rep.* (2020) 10:3007. doi: 10.1038/s41598-020-59940-1
63. Smulders TV. The avian hippocampal formation and the stress response. *Brain Behav Evol.* (2017) 90:81–91. doi: 10.1159/000477654
64. Herold C, Schlömer P, Mafoppa-Fomat I, Mehlhorn J, Amunts K, Axer M. The hippocampus of birds in a view of evolutionary connectomics. *Cortex.* (2019) 118:165–87. doi: 10.1016/j.cortex.2018.09.025
65. Taylor PS, Hemsworth PH, Groves PJ, Gebhardt-Henrich SG, Rault J. Frequent range visits further from the shed relate positively to free-range broiler chicken welfare. *Animal.* (2020) 14:138–49. doi: 10.1017/S1751731119001514
66. Guerrero-Bosagna C, Pértille F, Gomez Y, Rezaei S, Gebhardt S, Vögeli S, et al. DNA methylation variation in the brain of laying hens in relation to differential behavioral patterns. *Comp Biochem Physiol D.* (2020) 35:100700. doi: 10.1016/j.cbd.2020.100700
67. Vogeli SEA. The ranging behaviour of laying hens in relation to their personality. In: *51st International Congress of the ISAE*. Aarhus (2017).
68. Dunn IC, Wilson PW, Smulders TV, Sandilands V, D'eath RB, Boswell T. Hypothalamic agouti-related protein expression is affected by both acute and chronic experience of food-restriction and re-feeding in chickens. *J Neuroendocrinol.* (2013) 25:920–8. doi: 10.1111/jne.12088
69. Grigor PN, Hughes BO, Appleby MC. Effects of regular handling and exposure to an outside area on subsequent fearfulness and dispersal in domestic hens. *Appl Anim Behav Sci.* (1995) 44:47–55. doi: 10.1016/0168-1591(95)00576-E
70. Hartcher KM, Hickey KA, Hemsworth PH, Cronin GM, Wilkinson SJ, Singh M. Relationships between range access as monitored by radio frequency identification technology, fearfulness, and plumage damage in free-range laying hens. *Animal.* (2016) 10:847–53. doi: 10.1017/S1751731115002463
71. Larsen H, Hemsworth PH, Cronin GM, Gebhardt-Henrich SG, Smith CL, Rault J-L. Relationship between welfare and individual ranging behaviour in commercial free-range laying hens. *Animal.* (2018) 12:2356–64. doi: 10.1017/S1751731118000022
72. Drew M, Denny C, Hen R. Arrest of adult hippocampal neurogenesis in mice impairs single-but not multiple-trial contextual fear conditioning. *Behav Neurosci.* (2010) 124:a0020081. doi: 10.1037/a0020081
73. Anacker C, Hen R. Adult hippocampal neurogenesis and cognitive flexibility—linking memory and mood. *Nat Rev Neurosci.* (2017) 18:335–46. doi: 10.1038/nrn.2017.45
74. Drew LJ, Kheirbek MA, Luna VM, Denny CA, Cloidt MA, Wu MV, et al. Activation of local inhibitory circuits in the dentate gyrus by adult-born neurons. *Hippocampus.* (2016) 26:763–78. doi: 10.1002/hipo.22557
75. Anacker C, Luna VM, Stevens GS, Millette A, Shores R, Jimenez JC, et al. Hippocampal neurogenesis confers stress resilience by inhibiting the ventral dentate gyrus. *Nature.* (2018) 559:98–102. doi: 10.1038/s41586-018-0262-4
76. Fabel K, Wolf SA, Ehninger D, Babu H, Leal-Galicia P, Kempermann G. Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis in mice. *Front Neurosci.* (2009) 3:50. doi: 10.3389/neuro.22.002.2009
77. Ferreira VHB, Barbarat M, Lormant F, Germain K, Brachet M, Lovlie H, et al. Social motivation and the use of distal, but not local, featural cues are related to ranging behaviour in free-range chickens (*Gallus gallus domesticus*). *Anim Cognition.* (2020) 23:769–80. doi: 10.1007/s10071-020-01389-w
78. Tanti A, Rainer Q, Minier F, Surget A, Belzung C. Differential environmental regulation of neurogenesis along the septo-temporal axis of the hippocampus. *Neuropharmacology.* (2012) 63:374–84. doi: 10.1016/j.neuropharm.2012.04.022
79. Konefal S, Elliot M, Crespi B. The adaptive significance of adult neurogenesis: an integrative approach. *Front Neuroanat.* (2013) 7:21. doi: 10.3389/fnana.2013.00021
80. Ladage LD, Roth TC, Pravosudov VV. Hippocampal neurogenesis is associated with migratory behaviour in adult but not juvenile sparrows (*Zonotrichia leucophrys ssp.*). *Proc Royal Soc B.* (2010) 278:138–43. doi: 10.1098/rspb.2010.0861
81. Patel SN, Clayton NS, Krebs JR. Spatial learning induces neurogenesis in the avian brain. *Behav Brain Res.* (1997) 89:115–28. doi: 10.1016/S0166-4328(97)00051-X
82. Kempermann G, Brandon EP, Gage FH. Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. *Curr Biol.* (1998) 8:939–42. doi: 10.1016/S0960-9822(07)00377-6
83. Steiner B, Zurborg S, Hörster H, Fabel K, Kempermann G. Differential 24 h responsiveness of Prox1-expressing precursor cells in adult hippocampal neurogenesis to physical activity, environmental enrichment, and kainic acid-induced seizures. *Neuroscience.* (2008) 154:521–9. doi: 10.1016/j.neuroscience.2008.04.023

84. Tanti A, Westphal WP, Girault V, Brizard B, Devers S, Leguisquet AM, et al. Region-dependent and stage-specific effects of stress, environmental enrichment, and antidepressant treatment on hippocampal neurogenesis. *Hippocampus*. (2013) 23:797–811. doi: 10.1002/hipo.22134
85. Hawley DF, Leasure JL. Region-specific response of the hippocampus to chronic unpredictable stress. *Hippocampus*. (2012) 22:1338–49. doi: 10.1002/hipo.20970
86. Lehmann ML, Brachman RA, Martinowich K, Schloesser RJ, Herkenham M. Glucocorticoids orchestrate divergent effects on mood through adult neurogenesis. *J Neurosci.* (2013) 33:2961–72. doi: 10.1523/JNEUROSCI.3878-12.2013
87. Lee K, Kim S, Kim S, Choi S, Shin Y, Park S, et al. Chronic mild stress decreases survival, but not proliferation, of new-born cells in adult rat hippocampus. *Exp Mol Med.* (2006) 38:44–54. doi: 10.1038/emm.2006.6
88. Van Bokhoven P, Oomen CA, Hoogendijk WJG, Smit AB, Lucassen PJ, Spijker S. Reduction in hippocampal neurogenesis after social defeat is long-lasting and responsive to late antidepressant treatment. *Eur J Neurosci.* (2011) 33:1833–40. doi: 10.1111/j.1460-9568.2011.07668.x
89. Castilla-Ortega E, Rosell-Valle C, Pedraza C, Rodríguez De Fonseca F, Estivill-Torrús G, Santín LJ. Voluntary exercise followed by chronic stress strikingly increases mature adult-born hippocampal neurons and prevents stress-induced deficits in "what–when–where" memory. *Neurobiol Learn Mem.* (2014) 109:62–73. doi: 10.1016/j.nlm.2013.12.001
90. Lay Jr DC, Fulton RM, Hester PY, Karcher DM, Kjaer JB, et al. Hen welfare in different housing systems. *Poult Sci.* (2011) 90:278–94. doi: 10.3382/ps.2010-00962
91. Singh M, Cowieson A. Range use and pasture consumption in free-range poultry production. *Anim Prod Sci.* (2013) 53:1202–8. doi: 10.1071/AN13199
92. Dawkins MS, Cook PA, Whittingham MJ, Mansell KA, Harper AE. What makes free-range broiler chickens range? *In situ* measurement of habitat preference. *Anim Behav*. (2003) 66:151–60. doi: 10.1006/anbe.2003.2172
93. Zeltner E, Hirt H. Factors involved in the improvement of the use of hen runs. *Appl Anim Behav Sci.* (2008) 114:395–408. doi: 10.1016/j.applanim.2008.04.007
94. Kremer T, Jagasia R, Herrmann A, Matile H, Borroni E, Francis F, et al. Analysis of adult neurogenesis: evidence for a prominent "non-neurogenic" DCX-protein pool in rodent brain. *PLoS ONE.* (2013) 8:e59269. doi: 10.1371/journal.pone.0059269
95. Nacher J, Crespo C, Mcewen BS. Doublecortin expression in the adult rat telencephalon. *Eur J Neurosci.* (2001) 14:629–44. doi: 10.1046/j.0953-816x.2001.01683.x
96. Liu Y, Beyer A, Aebersold R. On the dependency of cellular protein levels on mRNA abundance. *Cell.* (2016) 165:535–50. doi: 10.1016/j.cell.2016.03.014

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer PT declared a past co-authorship with the author SG-H to the handling editor.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 September 2020
doi: 10.3389/fvets.2020.543158

# Virtual Fence Responses Are Socially Facilitated in Beef Cattle

*Hamideh Keshavarzi*, Caroline Lee, Jim M. Lea and Dana L. M. Campbell*

*Agriculture and Food, Commonwealth Scientific and Industrial Research Organization (CSIRO), Armidale, NSW, Australia*

Group-living can be socially advantageous where the behavior of individuals may be modified by group members through socially facilitative processes. Virtual fencing contains cattle by providing audio and electrical signals via a neckband device. However, little is known about social influences on learning to appropriately respond to the virtual fence (VF) cues. This study aimed to determine whether cattle respond to the behavior of conspecifics during their initial interactions with a VF across 3 days. Sixty-four Angus steers, naïve to virtual fencing, were placed into 8 paddocks (8 animals/group), divided with a VF into two areas- an inclusion and exclusion zone. The animals received an audio cue if they approached the VF followed by an electrical pulse if they continued into the exclusion zone. The GPS and audio and electrical stimuli data were recorded. To quantify social facilitation, individual VF interactions were grouped into 179 "events" across 3 days; starting from when the first animal (leader) approached the VF. The responses of other animals were categorized as (1) followed the leader to move into the exclusion zone (followers, F), (2) accompanied the leader back into the inclusion zone (facilitated, Fa), (3) did not show any reaction (non-facilitated, NFa). A social facilitation score (SFaS) was calculated as SFaS (%) = (F/(Fa+NFa+F)) * 100. A single leader animal led on average 37% of events with 76.2% of all reactions categorized as facilitated by other individuals. Animals responded to the behavior of conspecifics more during the VF implementation compared with facilitated movement during natural grazing patterns when no VF was present ($P < 0.001$). On average, cattle stopped or turned away to 3.8 (± 2.9 SE) audio cues before ever receiving their first electrical pulse. There was a positive correlation ($R = 0.34$, $P = 0.006$) between the number of audio cues received prior to the first electrical pulse and the proportion of all audio cues that were not followed by an electrical pulse. In conclusion, cattle stayed within the inclusion zone based on the response of conspecifics, including some social impacts on individual rates of associative learning between the audio and electrical cues.

**Keywords: facilitation, group-living, GPS, behavior, allelomimicry**

OPEN ACCESS

***Edited by:***
*Christian Nawroth, Leibniz Institute for Farm Animal Biology (FBN), Germany*

***Reviewed by:***
*Maria Vilain Rørvang, Swedish University of Agricultural Sciences, Sweden*
*Joao H. C. Costa, University of Kentucky, United States*

****Correspondence:***
*Hamideh Keshavarzi*
*hamideh.keshavarzi@csiro.au*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *16 March 2020*
***Accepted:*** *24 August 2020*
***Published:*** *30 September 2020*

***Citation:***
*Keshavarzi H, Lee C, Lea JM and Campbell DLM (2020) Virtual Fence Responses Are Socially Facilitated in Beef Cattle. Front. Vet. Sci. 7:543158. doi: 10.3389/fvets.2020.543158*

## INTRODUCTION

Social animal species live in groups which is thought to have several advantages for predator protection, improved foraging success (1) and may confer other social benefits such as keeping warm, mate access (2), allo-grooming (3, 4), and improved reproduction through maternal kinship (5). Although some individuals may move away from the group or vary in their proximity to other individuals (6) in group-living animals, there are collective processes occurring and the individuals

operate under consensus decisions (7). That is, while all animals are acting autonomously, they typically follow one or a few leaders resulting in coordinated group movements (8, 9). The influence of animals on moving group members into new areas can be related to their dominance status, age, or position in a social network (10, 11). Animal species in groups can also be influenced by conspecifics through watching or interacting with other individuals which can facilitate choosing what food to eat, or specifically how to access it, and predator avoidance (12, 13). There are multiple types of defined processes regarding the social transmission of behavior and information with varying degrees of evidence across different livestock species [reviewed in (14)]. The process of social facilitation (also called "allelomimicry" or "contagious behavior") is a term commonly used to define a situation where the behavior of one individual instigates the same behavior in another individual (14). Social facilitation is in contrast with social learning where an individual is stated to have socially learned a new behavior if it is retained when the demonstrator is absent (14).

Cattle typically live in groups of differing sizes, both in rangeland environments and more intensive farm herds. Within these groups there is evidence for social relationships between individuals (15, 16), differences in dominance status (6, 10), leaders and followers during grazing movements (11), and effects of social rank on milking patterns in automatic milking systems (17, 18) and positions at feed troughs (19). Cattle will demonstrate social facilitation (or allelomimicry) of postural behaviors such as greater synchronization of lying between neighboring individuals within a group (20) and synchronization of time budgets of different cattle breeds at pasture (21). Cattle will also show synchronized drinking behavior (22) and will graze specific toxic weeds if placed in paddocks with other cattle that readily consume them, including modifying previous correct aversions to the toxic plant (23, 24). The influence of social facilitation could thus be extended to other contexts of cattle farming such as the acclimation to and learning of new technologies.

In modern farming practices, new automated technologies such as automatic milking systems have changed livestock management (25). Livestock are expected to learn and respond appropriately to new farming environments and technologies (26) but learning may not be equal between all individuals resulting in culling of animals that do not adapt (27). Automated virtual fencing (VF) is a new agricultural technology that may transform the grazing livestock industry. Animals are restricted in a specified area via receiving stimulatory cues rather than through the presence of a physical fence (28) enabling remote animal monitoring and movement control. In the eShepherd® system (Agersens, Melbourne, VIC) all cattle wear a neckband device that will administer an audio tone as the animal approaches the VF, and an electrical stimulus if the animal continues moving forward. Cattle exposed to a VF show two stages of learning to avoid receiving electrical stimuli. Firstly, the cattle show avoidance learning where they rapidly learn to stay within the specified inclusion zone rather than continuing to move farther into the exclusion zone where they receive repeated audio/pulse combinations. This is followed by associative learning where they learn to respond appropriately to the audio cue alone (29, 30). However, individual cattle within the groups vary greatly in their rates of both avoidance and associative learning (29, 30) which could impact their adaptation to the technology (31). This individual variation may in part be a result of social influence. Campbell et al. (29, 30) found that cattle exposed to a VF for the first time learned to stay within the inclusion zone and respond to the audio cue alone, however, some cattle turned away from the audio cue without having first experienced the electrical stimulus suggesting social facilitation was occurring. It is currently unclear how social factors may affect cattle responses to a VF system. If cattle interact with the VF as a group during the initial stages of exposure, then social facilitation may improve the responses of some individuals resulting in 100% herd adaptation to the technology where all animals correctly remain in the inclusion zone. Alternatively, social facilitation may result in only some animals (leaders) being required to wear the neckband devices.

The current study aimed to look at the pattern of social facilitation during the first 3 days of VF activation by (1) identifying individuals that first approached the fence (leaders) within the groups, and (2) quantifying the degree of social facilitation when avoiding the VF boundary.

# MATERIALS AND METHODS

## Ethical Statement

The experiment was approved by the CSIRO FD McMaster Laboratory Chiswick Animal Ethics Committee (ARA18/25).

### Experimental Design

For this study, the data collected across 3 days each from 8 groups of eight 12 to 14-month old Angus steers (n = 64 animals) with an average starting body weight of 405 ± 31.8 kg were used. All cattle were naïve to virtual fencing for the data collection period of the current study. Data were collected from the 8 groups in a staggered method across a period of 3 months as there were limited numbers of both neckband devices and available paddocks to test all groups simultaneously. Additionally, groups 1–4 were part of a larger trial assessing the behavior and welfare of cattle exposed to electric tape or virtual fences conducted at the CSIRO Chiswick site in Armidale, NSW from January through March 2019 and full details of that experimental protocol can be found in Campbell et al. (29). This study used data from the first 3 days of VF exposure during the larger trial for groups 1 to 4 and is referred to as the "first trial." Groups 5–8 were those cattle that were exposed to electric tape during the larger trial but were then exposed to a VF for the first time 3 days immediately following the conclusion of the larger trial and these groups are referred to as the "second trial." Briefly, all cattle were fitted with eShepherd® neckbands that carried the virtual fencing device. Animals were placed into separate paddocks 9–14 ha in size with groups 1 and 2 placed in January 2019, groups 3 and 4 in February 2019, and groups 5–8 in March 2019. Paddocks were grassed with a tree line at one edge of 7 of the 8 paddocks as indicated in **Figure 1**. The average (mean ± SD) temperature (T) and relative humidity (RH) across the trial period (3 days)

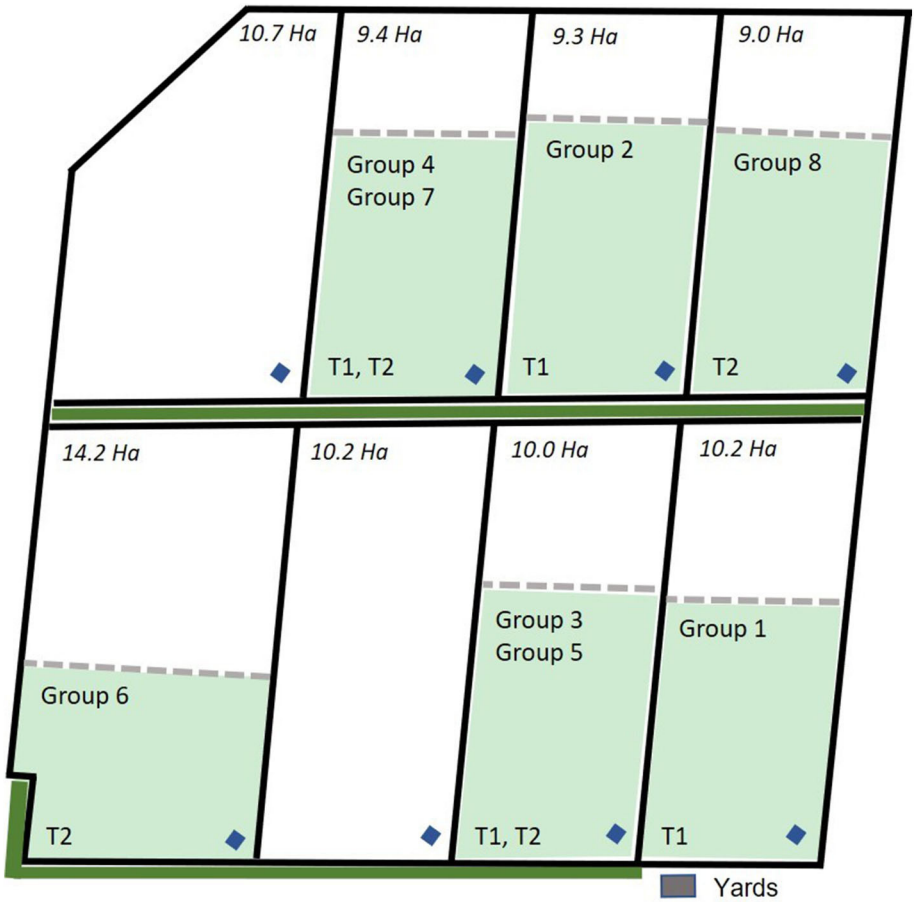


**FIGURE 1 |** Map of the group allocation to the paddocks across the trial period for groups 1 to 4 that were included in the first trial (T1) and groups 5 to 8 that were included in the second trial (T2). Paddock size (ha), location of the yards, water points (blue diamonds), tree line (solid green line) outside the paddock physical fences, and the placement of the virtual fence (dashed line) is indicated. Each fenced inclusion zone was 6 ha in size. Map is adapted from Campbell et al. (29).

were: T = 21.43 ± 0.41°C, RH = 72.76 ± 2.92% for groups 1 and 2; T = 16.50 ± 0.65°C, RH = 72.43 ± 2.45% for groups 3 and 4; T = 16.73 ± 3.21°C, RH = 76.83 ± 9.71% for groups 4 to 8 based on weather data collected directly at the Chiswick site.

All groups had an adaptation period to the paddocks with free access to the entire paddock area for 9 days. However, the groups in the second trial (groups 5–8) were placed back into the test paddocks and a virtual fence line was set the following morning to commence data collection. This timeline was selected as two of the groups had just spent the past 5 weeks in the paddock and the other two groups had previously spent 5 weeks in the paddocks as the electric-tape exposed groups from the larger trial presented in Campbell et al. (29). Following adaptation, single, straight, virtual fence boundaries were specified using GPS coordinates, and each paddock was divided into two areas—inclusion and exclusion zones. The inclusion zone was ∼6 ha in size across all paddocks (**Figure 1**). GPS coordinates of the virtual fence were transmitted to the unit using a radio frequency link. The animals received the audio cue if they approached the virtual fence. After receiving the audio cue, the animals could respond to the audio cue and stop and/or turn back to the inclusion area, or continue moving forward, in which case they received a short sharp pulse through the unit [further descriptions of the virtual fencing algorithm are reported in (29, 30, 32, 33)]. This sequence of an audio cue followed by the electrical pulse was repeated if the animal walked through the fence line and continued into the exclusion zone, but all cues ceased when an animal turned around to walk back out of the exclusion zone. The device had a safety limit for the number of consecutive pulses an animal received if it was continuing to move farther into the exclusion zone or it was moving above a specified velocity (i.e., running) but precise details on these functions are commercial in confidence. The device also included a "grazing function" to account for animals that may gradually encroach upon the VF by grazing. The natural behavioral pattern of grazing can mimic the correct response by the animal to the neckband cues where they may stop after receiving an audio cue during their slow grazing movement forward. Therefore, if an animal gradually moved into the exclusion zone and was not turning around when it received the audio signal, after 3 consecutive audio cues an electric pulse was applied. Each group

of 8 animals was exposed to the virtual fence for 3 days which was the sampling period for this study [this was the minimum exposure time for groups 1–4 as their responses to a VF were recorded for 4 weeks as part of (29)].

## Data

The time-stamped GPS positional data which recorded approximately every second when the animal was near the fence line and/or walking/grazing were downloaded from the neckband device. All audio cue and electrical pulse data of individual animals across 3 days of fence activation were also downloaded from the neckband device. One day of GPS data from the last day of the habituation period for each group was also included for control comparisons of social facilitation of movement patterns when no VF was present. Data editing was carried out in the SQL server (34). To prepare the dataset for analysis of social facilitation via GPS movement patterns and cues received, the original VF dataset was edited to eliminate records of: (1) before the first animal moved into the exclusion area, and (2) data during the night as it was assumed visual contact during learning would be limited. Thus, across 3 study days, the data used were based on sunrise and sunset in Armidale at the time of investigation for each group as follows: from 6 a.m. to 8 p.m. (groups 1 and 2), from 6:45 a.m. to 7:30 p.m. (groups 3 and 4), and from 7 a.m. to 7 p.m. (groups 5–8). For the first day, the time of the first interaction with the VF in each group (varied from 8 a.m. to 3:30 p.m. across the groups) was considered the starting time point. The control dataset for each group was of the same time periods across 1 day prior to activation of the VF.

## Social Influences Analyses

### *Control observations*

To compare the degree of social facilitation of movement patterns in the absence of a VF the movement of each group was scanned every 30 min across 1 day (daylight period only) until an instance was identified where the animal at the front of the group (termed the "leader") turned back in a different direction. The movements of the other individuals were then observed for up to 5 min to identify reactors (R—those animals who followed the leader in the same direction) and non-reactors (NR—those animals who did not follow the leader's direction change). A period of up to 5 min was selected as this was the maximum duration of the majority of VF events (**Table 1**). A total of 12 instances were identified for each group across the day which resulted in a power analysis equal to 0.99 in total across all groups ($n = 96$) and 0.7 within groups ($n = 12$). This following behavior was quantified into a percentage "social following score" for each event using the below equation:

$$Social\ Following\ Score\ \ (SFoS,\ \%) = \left(\frac{Reactors}{Reactors + Non - reactors}\right) * 100$$

### *Leadership during VF events*

In this study, the term "leadership" is used to define the first animal(s) who interacted with the VF and received signals for each separate interaction event. To quantify leadership during fence interactions, the group movement behavior was plotted in R (35) using the "ggplot2 package" (36) for each of the 8 groups during their first experience with the VF to describe initial group reactions to the stimuli. Leadership for each subsequent interaction was then determined across separate VF events. An event started from first contact with the VF where an animal received an audio cue only or an audio cue followed by an electric pulse. The event duration was then defined as from the time when the first animal touched the fence and at least one other animal reacted to their interaction until either (1) all animals moved away in distance from the virtual fence and had no more interactions (2) a minimum of 10 min had elapsed between when the last animal interacted and the first animal interacted in a new event, or (3) all animals had turned away from the direction of the virtual fence and then turned back toward it. Each event lasted up to 15 min; only 12.8% of all events were between 10 and 15 min duration (**Table 1**) with 3 events reaching 15 min where either the cattle broke through the fence and ventured far into the exclusion zone, or cattle were continuing to interact with the fence and receive signals during the first day of exposure. Typically, the cattle were grouped together and thus more than one individual interacted with the fence sequentially. There were only a few instances ($n = 5$) where an isolated individual touched the fence and received a signal and no other animals were near it and these data were excluded from the analyses. Across the 3 days for the 8 groups, a total of 179 separate events were recorded and the leader animal (s) identified (**Table 1**).

**TABLE 1 |** The percentage summary of VF interaction events of different durations (min) for 8 cattle groups[a].

| | | Duration of events | | |
|---|---|---|---|---|
| **Study groups** | **Total events (*n*)** | **1–5 min (%)** | **6–10 min (%)** | **11–15 min (%)** |
| Group 1 | 36 | 58.3 | 36.1 | 5.6 |
| Group 2 | 16 | 56.3 | 37.5 | 6.2 |
| Group 3 | 7 | 71.4 | 14.3 | 14.3 |
| Group 4 | 27 | 44.4 | 40.8 | 14.8 |
| Group 5 | 19 | 68.4 | 26.3 | 5.3 |
| Group 6 | 16 | 50.0 | 25.0 | 25.0 |
| Group 7 | 30 | 50.0 | 33.3 | 16.7 |
| Group 8 | 28 | 42.9 | 42.8 | 14.3 |
| Total/Mean | 179 | 55.2 | 32.0 | 12.8 |

[a] *Total events for each group was calculated based on interactions with the VF across 3 study days.*

### *Social facilitation*

For analysis of social facilitation, first the behavior of other individuals relative to the leader (s) during each VF interaction event (excluding the first event) was quantified. After the leader's interaction, the other animals within the group might (1) follow the leader into the exclusion zone, (2) follow the leader back into the inclusion zone, or (3) act independently of all the leader's movements. The animals' reactions (defined as movement in a backward or forward direction relative to the VF) were

monitored for a time period up to 15 min (**Table 1** displays the durations of identified events). In total, 171 events (of a total 179 events as the first event for each group was excluded) for animals in all groups across 3 study days were plotted in the R "ggplot2 package" (36) to look at the individual movement direction and the group's behavior to quantify how many animals moved back into the inclusion zone as a result of receiving a cue themselves or from watching the others (socially-facilitated). The animals were considered to have been socially-facilitated if they moved back into the inclusion zone without receiving a signal themselves within that particular event and when at least some of them had had previous experience with the VF. Data to study the social facilitation of the VF, therefore, was limited to the second event onwards when at least some animals had experienced the VF. The responses of other animals in the group were assessed in terms of their movement pattern (heading forward or turning back) within the group relative to the VF and position relative to other animals to indicate if they were staying within the inclusion zone based on the leader animals' interaction with the VF. In summary for each event the animals were considered as below:

Leader (s): The animal (s) who touched the VF first and received the signal as an audio cue or audio cue/electric pulse combination for each event.
Follower (s): The animal (s) who followed the leader to touch the VF/move into the exclusion zone with a time interval of at least 1 min after the leader(s) touched the VF.
Facilitated: The animal (s) who moved back into the inclusion zone as a result of accompanying the leader and his followers moving back into the inclusion zone without receiving any signal themselves.
Non-facilitated: The animal (s) who were close to the leader and his followers but did not change their movement direction to accompany them back into the inclusion zone nor did they interact with the VF.

Social facilitation score (SFaS): This is defined in this study as the proportion of animals who moved back into the inclusion zone as a result of the behavior of others without receiving a signal for each event. This was calculated as per below:

$$Social\ Facilitation\ Score\ (SFaS\ \%) = \left(\frac{Facilitated}{(Facilitated + Non - Facilitated + Followers)}\right) * 100$$

The animal(s) who were clearly separated from the rest of the group (based on visual inspection of the GPS plots) or whose movement path was in the opposite direction of the leader and VF line (mean of 88 m away from the main cluster; range: 40–240 m) at the time of an event were not considered in the social facilitation score calculation. In addition, all animals in groups 3, 4, and 7 had experience with the VF during the first interaction i.e., all "facilitated animals" were 100% experienced from the second interaction onwards. While the range of experience with the VF for facilitated animals in the second interaction for other groups were: group 1 = 62.5%, group 2 = 28.5% (one animal in group 2 was far away from the others on the first day and was not considered in this calculation), group 5 = 25%, group 6 = 75%, and group 8 = 87.5%.

The SFaS during the VF events for each study group for those events that were up to 5 min duration was compared with the SFoS from the identified control events using a unpaired two-tailed *t*-test (due to unequal events numbers for the control and test periods for each group) with α set at 0.05. For the overall comparison of SFaS and SFoS in which the average of these two parameters for study groups was used, the comparison was performed using a paired *t*-test. The percentage values were arcsine-transformed to meet the assumption of normality, but the raw values are presented in the results. In addition to quantifying the social facilitation during avoidance of the VF, the number of audio cues each animal received prior to receiving their first electric pulse were calculated to determine how social facilitation affected the associative learning between the audio cue and electrical pulse. These were calculated across the full dataset (including nighttime hours).

Finally, a Spearman correlation between the number of received audio cues before the first pulse and the proportion of "audio-only" cues (i.e., the proportion of all received audio cues that were not followed by a pulse) across 3 study days (nighttime also included) across each individual animal was estimated using the "ggpubr" package in R (37).

# RESULTS

## Leadership During VF Events

**Figure 2** presents the pattern of moving into the exclusion zone for the first time after the VF was activated for all studied groups. Overall, animals in each group behaved differently in whether they followed the leader animal (s) to move farther into the exclusion zone or back into the inclusion zone. For instance, all animals in groups 3, 4, and 7 received signals during the first interaction with the VF but animals in group 7 responded to the received signals by turning back into the inclusion zone while those in groups 3 and 4 broke the fence and moved farther into the exclusion zone, returning to the inclusion zone 10 min later. For the rest of the groups, the percentage of animals who received signals during the first interaction with the VF varied from 12.5% in group 2 (only the leader touched the fence at the first interaction) to 62.5% in group 5 (**Figure 2**).

**Table 2** presents the percentage contributions of leader animals across 3 study days in the 8 investigated groups. The information is presented in terms of total events that occurred and the percentage of events led by leader animals (one, two, or three) over the study period. Overall, one leader animal (alone or as a part of up to 3 leaders) in all groups led on average, 37% (varied from 27.8 to 71.4%) of events. Increasing the number of leader animals to two and three leaders covered 59.5% (varied from 42.8 to 100%), and 74.8% (varied from 60.7 to 100%) of events, respectively. The variance between groups in number of VF interactions (varied from 7 to 36 in the first trial vs. 16 to 30 in the second trial), and leadership contribution (e.g., varied from 31.2 to 71.4% in the first trial with one leader vs. 31.2 to 43.3% in the second trial with one leader) was less in the second trial compared to the first one (**Table 2**).

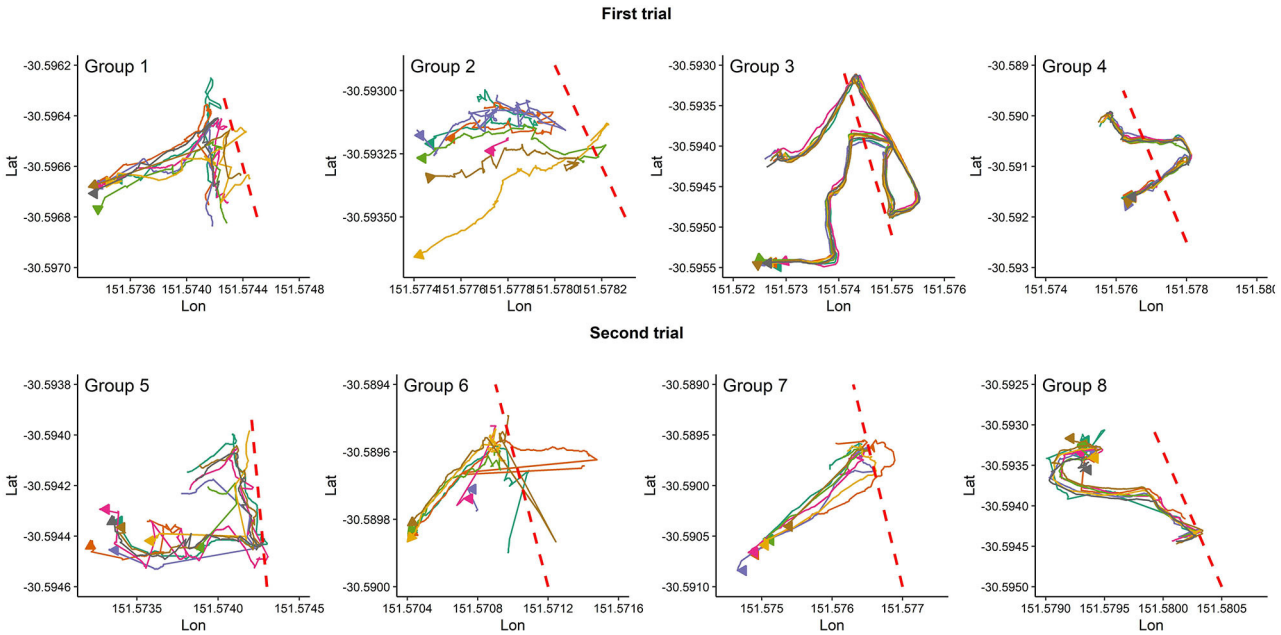


**FIGURE 2 |** Individual animal movement behavior during the first time interacting with the VF fence (red dashed line) for each group of 8 cattle. Plots are drawn across a period of 10 min, individual cattle are represented by separate colors, and the direction of the arrow indicates direction of travel at the conclusion of the 10 min period.

**TABLE 2 |** The percentage of virtual fence interaction events led by specific animals across 3 study days for 8 cattle groups.

| | | Events led by individual animals (%)[b] | | |
|---|---|---|---|---|
| **Groups[a]** | **Total events** | **One leader** | **Two leaders** | **Three leaders** |
| **First trial** | | | | |
| Group 1 | 36 | 27.8 (steer 3) | 52.8 (steer 3, 1) | 69.4 (steer 3, 1, 14) |
| Group 2 | 16 | 31.2 (steer 12) | 50.0 (steer 12, 11) | 68.7 (steer 12, 11, 13) |
| Group 3 | 7 | 71.4 (steer 24) | 100.0 (steer 24, 23) | 100.0 (steer 24, 23)[c] |
| Group 4 | 27 | 29.6 (steer 28) | 48.1 (steer 28, 19) | 69.9 (steer 28, 19, 31) |
| **Second trial** | | | | |
| Group 5 | 19 | 36.8 (steer 32) | 63.1 (steer 32, 37) | 84.2 (steer 32, 37, 13) |
| Group 6 | 16 | 31.2 (steer 2) | 56.2 (steer 2, 19) | 68.7 (steer 2, 19, 36) |
| Group 7 | 30 | 43.3 (steer 26) | 63.3 (steer 26, 3) | 76.7 (steer 26, 3, 12) |
| Group 8 | 28 | 25.0 (steer 24) | 42.8 (steer 24, 25) | 60.7 (steer 24, 25, 9) |
| Mean | 22.4 | 37.0 | 59.5 | 74.8 |

[a] *Groups 1–4 belonged to the first trial (1, 2: cohort 1, and 3, 4: cohort 2) and groups 5–8 belonged to the second trial (single cohort).*
[b] *The leader animals were the individuals who touched the fence first for each particular event across 3 study days.*
[c] *Group 3 already reached 100% of events with only two leader animals.*

## Social Facilitation

A total of 171 events (without considering the first interaction with the VF for each group) across 3 days for 8 groups were identified. On average, 76.2% of animals avoided the VF based on the behavior of other individuals which varied from 72.8% in group 8 to 80.5% in group 3 (**Table 3**). The percentage of social facilitation (SFaS) and number of interaction events fluctuated across the groups. Except for animals in group 4 and group 6, VF interactions had decreased by day 3. Overall, animals in group 1 and group 3 had the most and fewest mean VF interactions, respectively (group 1 mean = 11, group 3 mean = 2; **Table 3**). In terms of social facilitation, the percentage of animals who avoided the VF based on other individuals' interactions increased at the end of the study in over half of the groups, but decreased for groups 2, 3, and 5 resulting in all groups having similar mean social facilitation percentages across the 3 study days. Overall, the SFoS during control events (mean = 52.6%) was significantly ($df = 7$, $t = -9.57$, $P < 0.001$) lower than the SFaS (mean = 80.5% for events with up to 5 min duration) during VF events but variation

**TABLE 3 |** The summary of VF interaction events and social facilitation percentage of animals across 3 study days for 8 cattle groups including control comparisons.

| | Study groups | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Items/day** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| **Event no.** | | | | | | | | |
| Day 1 | 9 | 7 | 3 | 5 | 10 | 5 | 8 | 12 |
| Day 2 | 20 | 5 | 2 | 13 | 3 | 1 | 15 | 10 |
| Day 3 | 6 | 3 | 1 | 8 | 5 | 9 | 6 | 5 |
| Total | 35 | 15 | 6 | 26 | 18 | 15 | 29 | 27 |
| SFoS[a] | 54.7 | 57.1 | 54.4 | 44.0 | 56.3 | 50.0 | 60.7 | 61.2 |
| **SFaS (%)[b]** | | | | | | | | |
| Day 1 | 69.6 | 91.2 | 100.0 | 73.4 | 73.0 | 59.0 | 75.6 | 70.9 |
| Day 2 | 77.4 | 57.0 | 70.0 | 68.1 | 84.1 | 100.0 | 71.7 | 72.4 |
| Day 3 | 88.7 | 79.4 | 71.4 | 82.9 | 69.8 | 70.0 | 78.6 | 75.2 |
| Mean[c] | 78.6 | 75.9 | 80.5 | 74.8 | 75.6 | 76.3 | 75.3 | 72.8 |
| df[d] | 29 | 19 | 15 | 22 | 23 | 18 | 17 | 22 |
| *t*-value | −2.61 | −1.36 | −2.59 | −5.28 | −3.11 | −1.61 | −1.35 | −0.48 |
| *P*-value | 0.01 | 0.18 | 0.02 | <0.01 | 0.004 | 0.12 | 0.19 | 0.63 |

[a] *The social following score (SFoS) was calculated from control events as SFoS (%) = (R/(R+NR)) *100 where R (reactors) = animals who followed the leader in the same movement direction, and NR (non-reactors) = animals who did not follow the leader.*

[b] *The social facilitation score (SFaS) was calculated from VF events as SFaS (%) = (Fa/(Fa+NFa+F)) *100 where Fa (facilitated) = animals who accompanied the leader back into the inclusion zone, NFa (non-facilitated) = animals who did not show any reaction in terms of following the leader to move into the exclusion zone or accompanying him back into the inclusion zone, and F (followers) = animals who followed the leader into the exclusion zone.*

[c] *SFoS from control events up to 5 min duration was compared with the SFaS across all 3 study days with VF events that were up to 5 min.*

[d] *Degrees of freedom.*

*The first event was not considered in this table.*

in the SFoS/SFaS difference was present across the 8 groups (**Table 3**).

**Figure 3** illustrates some randomly selected examples of social facilitation during VF interaction events for animals in the first trial when at least some animals had experience with the VF. For instance, when animal 10 in group 2 or animal 5 in group 1 moved back into the inclusion zone following their received cues, other animals, even those with no experience with the VF (e.g., animals 9, and 16 in group 1; **Figure 3**) also moved back into the inclusion zone. In contrast, for the first interaction with the VF when no one had experience with the VF, almost all animals or those close (animals 11 and 13 in group 2) to the leader animal (s) followed him and moved into the exclusion zone (**Figure 4**).

Almost every animal in each group (63/64) responded correctly to the audio cues (i.e., stopped or turned away thus avoiding an electrical pulse) before ever receiving their first electrical pulse, but to different degrees ranging from 1 to 18 audio cues before the first pulse (**Table 4**). There was a positive significant correlation ($R = 0.34$, $P = 0.006$) between the number of audio cues received before the first pulse, and the proportion of "audio-only" cues across the 3 days (**Figure 5**).

## DISCUSSION

This study aimed to determine whether naïve beef cattle in small groups were socially facilitated in avoiding the exclusion zone in a virtual fencing (VF) system as well as in their associative learning between the audio and electrical cues. There were some individuals that first interacted with the VF more frequently, but there were no single animals that were consistently first within each group. Cattle showed clear patterns of social facilitation of movement behavior during VF interaction events where they stayed in the inclusion zone without receiving any cues themselves and turned away at the audio tone before ever experiencing the paired electrical pulse. This social facilitation of movement with a VF implemented was higher than movement facilitation during natural grazing patterns. This new evidence demonstrates that cattle can be influenced by each other during the implementation period of a novel agricultural technology.

The majority of interaction events with the VF occurred as a group with very few occasions where only one animal was by the fence line alone. This herding behavior is typical of cattle (6, 38) and enabled a group-level response to the VF where all animals stayed within the inclusion zone based on a few individuals receiving cues across each interaction event. That is, the studied cattle only had direct contact with the VF cues for 23.8% of the time, while for almost three quarters of the time they used the experience of other individuals to avoid the VF. However, there were no single individuals that always initiated the VF interactions. Some animals were more likely to be first to interact than others but with inconsistent patterns. Approximately 75% of events on average were led by three individuals within each group with more consistent patterns in some groups over others. These results are similar to previous observations of a single group of cattle exposed to a VF across a 10-day period where the frequency of being first to interact with the VF varied across individual cattle (30).

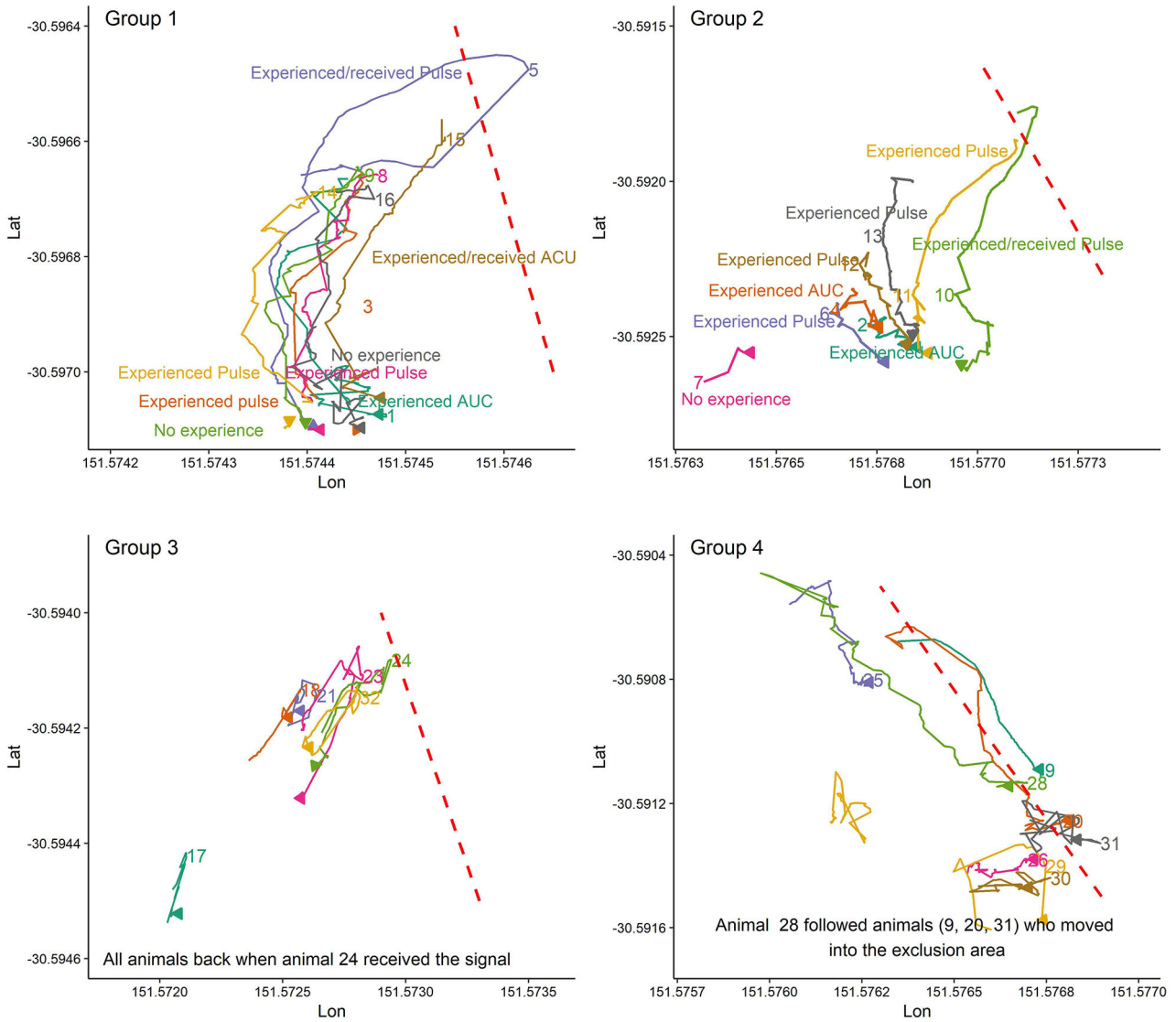


**FIGURE 3 |** The pattern of avoidance of the VF and exclusion zone as facilitated by others who reacted to the received cues and moved back into the inclusion zone when some animals had, and some animals did not have experience with the VF. Plots are drawn across a period of 5 min.

Some previous research has reported that dominant animals preferentially lead groups, such as when moving to new pasture areas (10). However, the relationship is typically non-linear, and no single individual shows exclusive herd leadership (9–11). This is consistent with the findings of the current study, but our assessment of leadership was restricted to one context. There was no assessment of the degree of influence these specific individuals may have had on group behavior in other situations (e.g., movement to a new grazing area, or movement to the water trough) and the relationship between VF leadership and dominance was not measured in these cattle. Thus, the animals were not identified as consistent leaders of the group but only as individuals of influence at the time of the interaction (39), limiting the conclusions regarding the influence that specific individuals may have on the behavior of other group members. Classification of dominance and quantification of the social dynamics within the group may provide further insight into patterns of facilitation where individuals differing in status, age, or social position may exert more influence on the group [e.g., in cattle (11); in chickens (40)]. Differences in some of these parameters could also account for the variation that was seen between groups in both the consistency of first interactors and degree of social facilitation (**Tables 2**, **3**). Alternatively, personality differences may have affected interactions with the VF where bolder animals with higher motivation to explore and/or access the area in the exclusion zone may have initiated more VF interactions (41, 42). Regardless of the reasons for these individual differences, for VF technology to be successfully implemented, all cattle should wear neckband devices as no single individual of influence was identified.

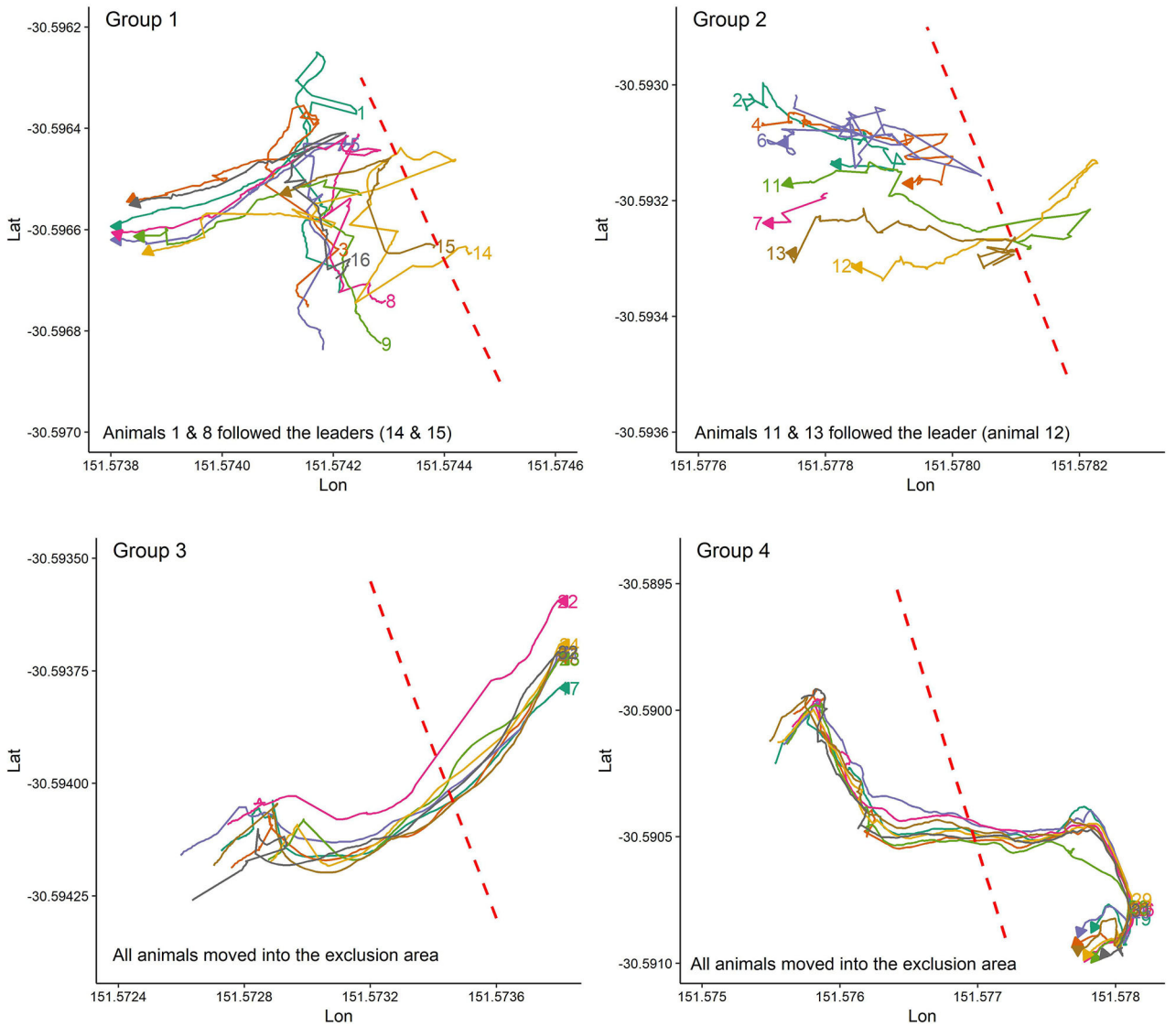


**FIGURE 4 |** The animal's reactions during the first contact with the VF when animals have no experience with the VF. Plots are drawn across a period of 5 min.

The findings from this study show that adapting to a new technology can be facilitated by conspecifics when exposed to the VF system as a group. Previous studies of individual associative learning patterns (between the audio cue and electrical stimulus) during first exposure to the VF cues and avoidance learning (remaining within the inclusion zone) demonstrated high variation in both the rate of learning and the behavioral responses to the cues with some individuals running forward following an electrical pulse, and other animals turning away (28). When exposed as a group, the behavioral responses to the VF are more cohesive (i.e., all members of a group act in a similar manner), although they still vary between separate groups. This variation was particularly apparent in the very first experience with the VF where some groups of animals all received signals and broke through into the exclusion zone, compared with other groups that all turned around based on the experiences of only a few animals.

Facilitated or synchronized responses are typical of cattle (20–22) and were also shown in the current study during the control observations of natural grazing patterns. However, overall, comparatively more facilitation of movement was observed when the VF was implemented. Group-level responses in a potentially threatening situation are one of the benefits of group-living (43). Other research has shown cattle will act as a coordinated group in their patterns of avoiding biting pests (44, 45). In the case of the VF, the stimuli are initially highly unexpected with no visual cues and a benign audio tone as a warning for the electrical pulse. Avoidance based on the avoidance reactions of others can initially minimize an individual's experience of what may be observed as a negative experience of conspecifics

**TABLE 4 |** Number of received audio cues before the first electrical pulse for individuals (1–8) in the studied groups.

| | Received audio cue/Animals' number* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Trial/group** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **Mean/group** |
| **First trial** | | | | | | | | | |
| Group 1 | 18 (1) | 3 (3) | 1 (5) | 3 (8) | 2 (9) | 3 (14) | 6 (15) | 3 (16) | 4.9 |
| Group 2 | 3 (2) | 9 (4) | 3 (6) | 12 (7) | 3 (10) | 4 (11) | 3 (12) | 1 (13) | 4.8 |
| Group 3 | 3 (17) | 4 (18) | 2 (21) | 4 (22) | 3 (23) | 3 (24) | 5 (27) | 1 (32) | 3.1 |
| Group 4 | 3 (19) | 1 (20) | 4 (25) | 3 (26) | 5 (28) | 1 (29) | 2 (30) | 1 (31) | 2.5 |
| **Second trial** | | | | | | | | | |
| Group 5 | 1 (6) | 3 (13) | 6 (17) | 1 (22) | 1 (29) | 3 (30) | 8 (32) | 4 (37) | 3.4 |
| Group 6 | 4 (2) | 5 (14) | 4 (19) | 2 (20) | 4 (35) | 3 (36) | 3 (38) | 3 (39) | 3.5 |
| Group 7 | 3 (3) | 3 (10) | 1 (12) | 3 (16) | 7 (18) | 3 (26) | 9 (27) | – | 4.1 |
| Group 8 | 3 (4) | 3 (5) | 1 (7) | 4 (9) | 2 (23) | 9 (24) | 5 (25) | 4 (34) | 3.9 |

**The numbers in parentheses are the ID of animals in each group.*

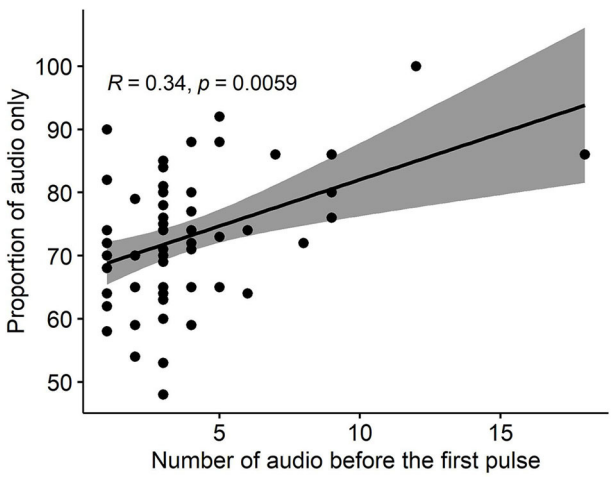


**FIGURE 5 |** The correlation between the number of received audio cues before the first pulse and the proportion of "audio-only" cues (i.e., the proportion of all received audio cues that were not followed by a pulse) across 3 study days for all groups.

when they suddenly react to the electrical pulse. The degree of reactivity by individuals receiving a pulse vs. unknown stimuli that instigated a change in direction during grazing likely resulted in the comparatively heightened facilitation of group members behavior in the presence of a VF (46). Subsequently, avoidance based on herd member's reactions can also minimize the frequency of moving into the exclusion zone and receiving electrical pulses. However, the VF eShepherd® system has been designed to be controllable and predictable for all individuals if they learn the association between the audio cue and electrical pulse (31). Through associative learning, all individuals can avoid receiving electrical pulses if they appropriately stop or turn away at the audio tone. In this study we demonstrated that individuals responded correctly to the audio cue multiple times without ever receiving an electrical pulse, indicating they were avoiding a benign stimulus, based on observations of conspecifics. The VF devices are designed to emit audio tones at a decibel level audible only to the animal wearing the device, although in calm conditions and close proximity, audio tones could potentially be heard by neighboring animals. It is thus likely that the cattle were associating their own audio tone with an avoidance response from watching the reactions of herd mates before they had received their own electrical stimulus for the first time. A similar observation has previously been stated for cattle learning to respond to a standard electric fence where animals avoided the fence without experiencing it themselves (47). In the current study, this facilitated response to the audio cues then resulted in some improvements in the rate of associative learning across the 3 day study duration but further research should elucidate the exact mechanisms behind this and whether the different types of learning (from watching others or self-experience) have any corresponding physiological and emotional impacts such as increased heart rate when learning is successful (48). The distinction between social facilitation and social learning and cognitive processes behind the observed behavioral patterns was unclear from the current study and future work should aim to determine the degree to which cattle may learn a VF system from observing others which could be achieved by exposure as a group followed by individual testing. Additionally, the number of assessed groups and group sizes were limited by available animals, paddocks, and pasture. Further testing across more groups and larger group sizes would confirm the degree to which successful implementation of a VF is influenced by social processes.

In conclusion, appropriately responding to virtual fencing technology is socially facilitated via observations of the reactions and behavior of other group members in beef cattle. In large commercial cattle groups, this could improve the effectiveness of the fence and minimize the number of electrical pulses each animal receives. Different animal groups vary in their behavioral reactions and learning rates, further assessment of group dominance hierarchies or social interactions may help understand the causes of these differences.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article may be made available by the authors to any qualified researcher, but only with the approval of Agersens Pvt. Ltd. to ensure commercial confidentially is maintained.

## ETHICS STATEMENT

This animal study was reviewed and approved by CSIRO FD McMaster Laboratory Chiswick Animal Ethics Committee (ARA18/25).

## AUTHOR CONTRIBUTIONS

CL, DC, HK, and JL contributed conception and design of the study, and organized the database. HK performed the statistical analyses and wrote the first draft of the manuscript. DC, HK, and CL wrote sections of the manuscript. All authors approved the final version.

## FUNDING

This project was supported by funding from the Australian Government Department of Agriculture, Water and the Environment as part of its Rural R&D for Profit program. HK was supported by a Commonwealth Scientific and Industrial Research Organization (CSIRO) Research Plus postdoctoral fellowship.

## ACKNOWLEDGMENTS

The authors are grateful to Troy Kalinowski (CSIRO) for technical support, Andrew Eichorn (CSIRO) for animal management support, and the team at Agersens for ongoing support with the eShepherd® system.

## REFERENCES

1. Rubenstein DI. On predation, competition, and the advantages of group living. In: Bateson PPG, Klopfer PH, editors. *Social Behavior. Perspectives in Ethology*. Vol. 3. Boston, MA: Springer (1978). p. 205–31.
2. Durand E, Blum MGB, François OO. Prediction of group patterns in social mammals based on a coalescent model. *J Theor Biol*. (2007) 249:262–70. doi: 10.1016/j.jtbi.2007.07.012
3. Bouissou MF, Boissy A, le Neindre P, Veissier I. The social behaviour of cattle. In: Keeling L, Gonyou F, editors. *Social Behaviour in Farm Animals*. Wallingform: CABI Publishing (2001). p. 113–45.
4. Schiano G. Grooming, competition and social rank among female primates: a meta-analysis. *Anim Behav*. (2001) 62:265–71. doi: 10.1006/anbe.2001.1750
5. Lynch EC, Lummaa V, Htut W, Lahdenperä M. Evolutionary significance of maternal kinship in a long-lived mammal. *Phil Trans R Soc. B.* (2019) 374:20180067. doi: 10.1098/rstb.2018.0067
6. Harris NR, Johnson DE, McDougald NK, George MR. Social associations and dominance of individuals in small herds of cattle. *Rangeland Ecol Manag*. (2007) 60:339–49. doi: 10.2111/1551-5028(2007)60[339:SAADOI]2.0.CO;2
7. Conradt L, Roper TJ. Consensus decision making in animals. *Trends Ecol Evol.* (2005) 20:449–56. doi: 10.1016/j.tree.2005.05.00
8. Sato S. Leadership during actual grazing in a small herd of cattle. *Appl Anim Ethol*. (1982) 8:53–65. doi: 10.1016/0304-3762(82)90132-8
9. Dumont B, Boissy A, Achard C, Sibbald AM, Erhard HW. Consistency of animal order in spontaneous group movements allows the measurement of leadership in a group of grazing heifers. *Appl Anim Behav Sci*. (2005) 95:55–66. doi: 10.1016/j.applanim.2005.04.005
10. Šárová R, Špinka M, Panamá JLA, Šimeček P. Graded leadership by dominant animals in a herd of female beef cattle on pasture. *Anim Behav*. (2010) 79:1037–45. doi: 10.1016/j.anbehav.2010.01.019
11. Sueur C, Kuntz C, Debergue E, Keller B, Robic F, Siegwalt-Baudin F, et al. Leadership linked to group composition in Highland cattle (*Bos taurus*): implications for livestock management. *Appl Anim Behav Sci*. (2018) 198:9–18. doi: 10.1016/j.applanim.2017.09.014
12. Galef BG, Laland KN. Social learning in animals: empirical studies and theoretical models. *BioScience*. (2005) 55:489. doi: 10.1641/0006-3568(2005)055[0489:SLIAES]2.0.CO;2
13. Reader SM. Animal social learning: associations and adaptations. *F1000Research*. (2016) 5:2120. doi: 10.12688/f1000research.7922.1
14. Nicol CJ. The social transmission of information and behaviour. *Appl Anim Behav Sci*. (1995) 44:79–98. doi: 10.1016/0168-1591(95)00607-T
15. Foris B, Zebunke M, Langbein J, Melzer N. Comprehensive analysis of affiliative and agonistic social networks in lactating dairy cattle groups. *Appl Anim Behav Sci*. (2019) 210:60–7. doi: 10.1016/j.applanim.2018.10.016
16. Gygax L, Neisen G, Wechsler B. Socio-spatial relationships in dairy cows. *Ethology*. (2009) 116:10–23. doi: 10.1111/j.1439-0310.2009.01708.x
17. Lauwere CCK, Devir S, Metz JHM. The influence of social hierarchy on the time budget of cows and their visits to an automatic milking system. *Appl Anim Behav Sci*. (1996) 49:199–211. doi: 10.1016/0168-1591(96)01030-1
18. Melin M, Hermans GGN, Pettersson G, Wiktorsson H. Cow traffic in relation to social rank and motivation of cows in an automatic milking system with control gates and an open waiting area. *Appl Anim Behav Sci*. (2006) 96:201–14. doi: 10.1016/j.applanim.2005.06.013
19. Manson FJ, Appleby MC. Spacing of dairy cows at a food trough. *Appl Anim Behav Sci*. (1990) 26:69–81. doi: 10.1016/0168-1591(90)90088-U
20. Stoye S, Porter MA, Dawkins MS. Synchronized lying in cattle in relation to time of day. *Livest Sci*. (2012) 149:70–3. doi: 10.1016/j.livsci.2012.06.028
21. Braghieri A, Pacelli C, Girolami A, Napolitano F. Time budget, social and ingestive behaviours expressed by native beef cows in Mediterranean conditions. *Livest Sci*. (2011) 141:47–52. doi: 10.1016/j.livsci.2011.05.001
22. Lardner HA, Braul L, Schwartzkopf-Genswein K, Schwean-Lardner K, Damiran D, Darambazar E. Consumption and drinking behavior of beef cattle offered a choice of several water types. *Livest Sci*. (2013) 157:577–85. doi: 10.1016/j.livsci.2013.08.016
23. Ralphs MH, Olsen JD. Adverse influence of social facilitation and learning context in training cattle to avoid eating larkspur. *J Anim Sci*. (1990) 68:1944–52. doi: 10.2527/1990.6871944x
24. Ralphs MH, Graham D, James LF. Social facilitation influences cattle to graze locoweed. *J Range Manag*. (1994) 47:123–6.
25. John AJ, Clark CEF, Freeman MJ, Karrisk KL, Garcia SC, Halachmi I. Review: milking robot utilization, a successful precision livestock farming evolution. *Animal*. (2016) 10:1484–92. doi: 10.1017/S1751731116000495
26. Wechsler B, Lea SEG. Adaptation by learning: Its significance for farm animal husbandry. *Appl Anim Behav Sci*. (2007) 108:197–214. doi: 10.1016/j.applanim.2007.03.012
27. Tse C, Barkema HW, DeVries TJ, Rushen J, Vasseur E, Pajor EA. Producer experience with transitioning to automatic milking: cow training, challenges, and effect on quality of life. *J Dairy Sci*. (2018) 101:9599–607. doi: 10.3168/jds.2018-14662
28. Campbell DLM, Lea JM, Haynes SJ, Farrer WJ, Leigh-Lancaster CJ, Lee C. Virtual fencing of cattle using an automated collar in a feed attractant trial. *Appl Anim Behav Sci*. (2018) 200:71–7. doi: 10.1016/j.applanim.2017.12.002

29. Campbell DLM, Lea JM, Keshavarzi H, Lee C. Virtual fencing is comparable to electric tape fencing for cattle behavior and welfare. *Front Vet Sci*. (2019) 6:445. doi: 10.3389/fvets.2019.00445
30. Campbell DLM, Haynes SJ, Lea JM, Farrer WJ, Lee C. Temporary exclusion of cattle from a riparian zone using virtual fencing technology. *Animals*. (2019) 9:5. doi: 10.3390/ani9010005
31. Lee C, Colditz IG, Campbell DLM. A framework to assess the impact of new animal management technologies on welfare: a case study of virtual fencing. *Front Vet Sci*. (2018) 5:187. doi: 10.3389/fvets.2018.00187
32. Lee C. *An Apparatus and Method for the Virtual Fencing of an Animal*. International Patent Application PCT/AUT2005/001056. International Publication No. WO 2006/007643 (2006).
33. Lee C, Reed M, Wark T, Crossman C, Valencia P. A *Control Device, and Method, for Controlling the Location of an Animal*. International Patent Application PCT/AU2009/000943. International Publication No. WO2010/009509 (2010).
34. Microsoft (2012). *Microsoft SQL Server Management Studio*. Available online at: http://www8.cs.umu.se/education/examina/Rapporter/EsquiviasFlarup.pdf (accessed July 29, 2020).
35. R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna (2015).
36. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag (2016). Available online at: https://ggplot2.tidyverse.org (accessed July 29, 2020).
37. Kassambara A. *ggpubr: 'ggplot2' Based Publication Ready Plots*. (2020). Available online at: https://cran.r-project.org/web/packages/ggpubr/index.html (accessed July 29, 2020).
38. Stephenson MB, Bailey DW, Jensen D. Association patterns of visually-observed cattle on Montana, USA foothill rangelands. *Appl Anim Behav Sci*. (2016) 178:7–15. doi: 10.1016/j.applanim.2016.02.007
39. Strangburg-Peshkin A, Papageorgiou D, Crofoot MC, Farine DR. Inferring influence and leadership in moving animal groups. *Phil Trans R Soc B*. (2018) 373:20170006. doi: 10.1098/rstb.2017.0006
40. Nicol CJ, Pope SJ. Social learning in small flocks of laying hens. *Anim Behav*. (1994) 47:1289–96. doi: 10.1006/anbe.1994.1177
41. Müller R, Schrader L. Behavioural consistency during social separation and personality in dairy cows. *Behaviour*. (2005) 142:1289–396. doi: 10.1163/156853905774539346
42. Müller R, von Keyserlingk MAG. Consistency of flight speed and its correlation to productivity and to personality in *Bos taurus* beef cattle. *Appl Anim Behav Sci*. (2006) 99:193–204. doi: 10.1016/j.applanim.2005.05.012
43. Olson RS, Haley PB, Dyer FC, Adami C. Exploring the evolution of a trade-off between vigilance and foraging in group-living organisms. *R Soc Open Sci*. (2015) 2:150135. doi: 10.1098/rsos.150135
44. El Ashmawy WR, Williams DR, Gerry AC, Champagne JD, Lehenbauer TW, Aly SS. Risk factors affecting dairy cattle protective grouping behavior, commonly known as bunching, against *Stomoxys calcitrans* (L.) on California dairies. *PLoS ONE*. (2019) 14:e224987. doi: 10.1371/journal.pone.0224987
45. Ralley WE, Galloway TD, Crow GH. Individual and group behaviour of pastured cattle in response to attack by biting flies. *Can J Zool*. (1993) 71:725–34. doi: 10.1139/z93-096
46. Boissy A, Terlouw C, Le Neindre P. Presence of cues from stressed conspecifics increases reactivity to aversive events in cattle: evidence for the existence of alarm substances in urine. *Physiol Behav*. (1998) 63:489–95. doi: 10.1016/S0031-9384(97)00466-6
47. McKillop IG, Sibly RM. Animal behaviour at electric fences and the implications for management. *Mammal Rev*. (1988) 18:91–103. doi: 10.1111/j.1365-2907.1988.tb00078.x
48. Hagen K, Broom DM. Emotional reactions to learning in cattle. *Appl Anim Behav Sci*. (2004) 85:203–13. doi: 10.1016/j.applanim.2003.11.007


**Conflict of Interest:** Agersens Pty Ltd. provided technical support with the neckbands but did not contribute to the study design, data analysis, or the decision to publish the results.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 07 December 2020
doi: 10.3389/fvets.2020.583715



# Proximity Interactions in a Permanently Housed Dairy Herd: Network Structure, Consistency, and Individual Differences

*Kareemah Chopra[1], Holly R. Hodges[2], Zoe E. Barker[2†], Jorge A. Vázquez Diosdado[1†], Jonathan R. Amory[2], Tom C. Cameron[3], Darren P. Croft[4], Nick J. Bell[5†] and Edward A. Codling[1*]*

[1] Department of Mathematical Sciences, University of Essex, Colchester, United Kingdom, [2] Writtle University College, Chelmsford, United Kingdom, [3] School of Life Sciences, University of Essex, Colchester, United Kingdom, [4] Centre for Research in Animal Behaviour, College of Life and Environmental Sciences, University of Exeter, Exeter, United Kingdom, [5] Royal Veterinary College, Hatfield, United Kingdom

Understanding the herd structure of housed dairy cows has the potential to reveal preferential interactions, detect changes in behavior indicative of illness, and optimize farm management regimes. This study investigated the structure and consistency of the proximity interaction network of a permanently housed commercial dairy herd throughout October 2014, using data collected from a wireless local positioning system. Herd-level networks were determined from sustained proximity interactions (pairs of cows continuously within three meters for 60 s or longer), and assessed for social differentiation, temporal stability, and the influence of individual-level characteristics such as lameness, parity, and days in milk. We determined the level of inter-individual variation in proximity interactions across the full barn housing, and for specific functional zones within it (feeding, non-feeding). The observed networks were highly connected and temporally varied, with significant preferential assortment, and inter-individual variation in daily interactions in the non-feeding zone. We found no clear social assortment by lameness, parity, or days in milk. Our study demonstrates the potential benefits of automated tracking technology to monitor the proximity interactions of individual animals within large, commercially relevant groups of livestock.

**Keywords: animal group, animal movement, dairy cow, lameness, local positioning system (LPS), precision livestock farming (PLF), proximity interactions, social network analysis (SNA)**



***Edited by:***
*Dana L. M. Campbell, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia*

***Reviewed by:***
*Shenghong Li, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia*
*Oleksiy Guzhva, Swedish University of Agricultural Sciences, Sweden*
*Melissa Cantor, University of Kentucky, United States*

***Correspondence:**
*Edward A. Codling*
*ecodling@essex.ac.uk*

***†Present address:***
*Zoe E. Barker, School of Agriculture, Policy and Development, University of Reading, Reading, United Kingdom; Jorge A. Vazquez Diosdado, School of Veterinary Medicine and Science, University of Nottingham, Nottingham, United Kingdom; Nick J. Bell, Bos International Ltd., Wimborne, United Kingdom and School of Veterinary Medicine and Science, University of Nottingham, Nottingham, United Kingdom*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *15 July 2020*
***Accepted:*** *13 November 2020*
***Published:*** *07 December 2020*

***Citation:***
*Chopra K, Hodges HR, Barker ZE, Vázquez Diosdado JA, Amory JR, Cameron TC, Croft DP, Bell NJ and Codling EA (2020) Proximity Interactions in a Permanently Housed Dairy Herd: Network Structure, Consistency, and Individual Differences. Front. Vet. Sci. 7:583715. doi: 10.3389/fvets.2020.583715*

## INTRODUCTION

The herd social structure of cows on most commercial dairy farms differs significantly from their wild counterparts (1). Dairy cows are typically kept in exclusively female groups, separated by age and reproductive status, with access to a more restricted space allowance in the form of either indoor housing or fenced grazing paddocks and may be subject to frequent regrouping events (2–5). Understanding the structure and dynamics of housed dairy cattle networks may give insights on preferential interactions and aid in optimizing their management (6, 7).

The social structure of animal groups, including how associations and interactions between individuals change over time, can be assessed using social network analysis (SNA) (8). The approach

is well established; SNA is used across multiple disciplines including sociology (9), computer science (10), and transport (10, 11), and has been developed to study animal social networks, particularly over the last decade (12, 13). SNA has been used to explore interactions in dairy cattle, revealing highly clustered herds (14–16). Cows appear to associate non-randomly, potentially based on attributes such as lactation number (14, 15). Inter-individual variation in sociality has been found in dairy cattle, potentially driven by personality, established as consistent from calf to adulthood (except during puberty) (17), or dominance, as studied in (18) who found that some individuals are more influential than others within the social network. Housed cattle are known to avoid interactions with dominant conspecifics whilst feeding to reduce competition (19), and the social positioning of individuals may also be altered where a resource is deemed more valuable (20). Individual attributes are thought to be important in disease transmission (7), as cows participate in contact behaviors based on age and sex. Dairy cows may groom conspecifics based on familiarity and dominance (21), although affiliative and agonistic interaction networks may not be correlated (22).

Data can be collected for SNA in non-automated ways, such as through direct observation (7, 21) or through analysis of video recordings (22). Although detailed social interaction data can be obtained through these methods, they are highly time-consuming, and limit sample size and sampling duration. Developments in technology mean that it is now possible to collect absolute or relative spatial positioning data in an automated way using proximity sensors or positioning systems, recording detailed locations of all animals in the herd over time. Global positioning system (GPS) can be used to track cattle outdoors (23), but mean location errors are typically around 5 m in commercial systems and can be as high as 19.6 m (24). As GPS does not function indoors, alternative systems are needed for housed dairy cows, such as sensor-based local positioning systems (LPS), which have been validated with dairy cows with mean error typically around 2–3 m, although 0.5 m mean error may be achievable (15, 25–28). The simplest interaction networks are then developed by assuming interactions occur when two individuals are within a given proximity, usually based on metric distance, for a specified time duration (6, 8, 14, 29); while analysis based on topological distances (30) or more complex interactions and social dominance are also feasible (31).

Modern productions systems, while efficient, expose cattle to risks for several production diseases, including lameness, mastitis, and metabolic diseases. Lameness is a significant issue globally with average farm level prevalence estimates of 28–32% in Europe (32, 33), 28–39% in South America (34, 35) and 30–55% in North America (36). System related promoters of lameness include high yields (37, 38) driven by genetic selection, and nutrition and environmental factors such as increased standing time on unsuitable floor surfaces (39–41). Early detection of lameness and prompt treatment is essential to reduce its severity and duration (42, 43) and to prevent re-occurrence (44, 45). Under-estimation of lameness by farmers remains a problem which can lead to delays in treatment (46–48). To identify lame cows, farmers typically observe elements of a cow's gait, which is prone to error and largely subjective (49), and abnormal behaviors may not be immediately obvious (50). While on some farms this process may be formalized by scoring all cows against a recognized locomotion score (51), on many farms cows are only observed during routine tasks, increasing subjectivity and the risk of missing a large proportion of the herd. Precision Livestock Farming (PLF) techniques, where farm management is aided through continuous automated real-time monitoring of animals or the environment (24, 26, 27) provide opportunities to support rapid identification of lameness and other production diseases. Lameness has been associated with inflammatory responses (52) and results in generalized sickness behaviors which could be monitored using PLF techniques. Changes to individual cow behavior associated with lameness have also been investigated using PLF techniques to identify modified feeding and lying behavior (53–56), and space use (57). Sick cows are less likely to approach humans (58, 59), and both cows and calves have been observed to alter their positioning in a herd when ill (60–62). Evidence suggests cows with ketosis and mastitis displace conspecifics less frequently (63–65). Lame cows may alter their time budgets with lame individuals spending less time feeding than their healthy counterparts (53, 57). Lame cows also appear to be licked by conspecifics more than non-lame cows (66). Despite this existing evidence, to our knowledge automated PLF techniques have not been applied to monitor changes in social behavior in cattle that could be associated with disease.

In this study we investigate the structure and consistency of the proximity interaction network of a large permanently housed dairy herd using positional data collected from an automated local positioning system (LPS). We determine the level of inter-individual variation in proximity interactions across different functional zones of the barn (feeding, non-feeding) and assess how these interactions vary during the month-long study period. We consider the influence of health status (specifically lameness), parity, and days in milk (DIM), on the sociality and interactions of individuals within the herd.

# METHODOLOGY

## Animals and Housing

A high-yielding management group of Holstein-Friesian dairy cattle were observed continuously throughout October 2014 on a commercial farm in southeast England. Our study group consisted of 92 cows that were continuously present in the barn throughout the study duration (mean days in milk (DIM) = 136 and mean parity = 3). These cows formed part of a larger group (100 to 111 on any given day in the month, mean = 105, standard error = 0.59), with averages calculated from April 2013 to April 2014 of: calving interval of 416 days, 305 daily milk yield of 10,909 liters, 63% pregnant, somatic cell count of 140,000 cells/ml. Localized weather and temperature, which are known to affect behavior (67), were largely stable throughout the study period (mean range of 12.4–19.9 degrees Celsius). Cows were housed permanently indoors inside one half of a commercial free-stall barn containing 98 useable cubicles bedded with sawdust over mattresses (**Figure 1**). Central passageways allowed free movement around the barn and access to the central feeding

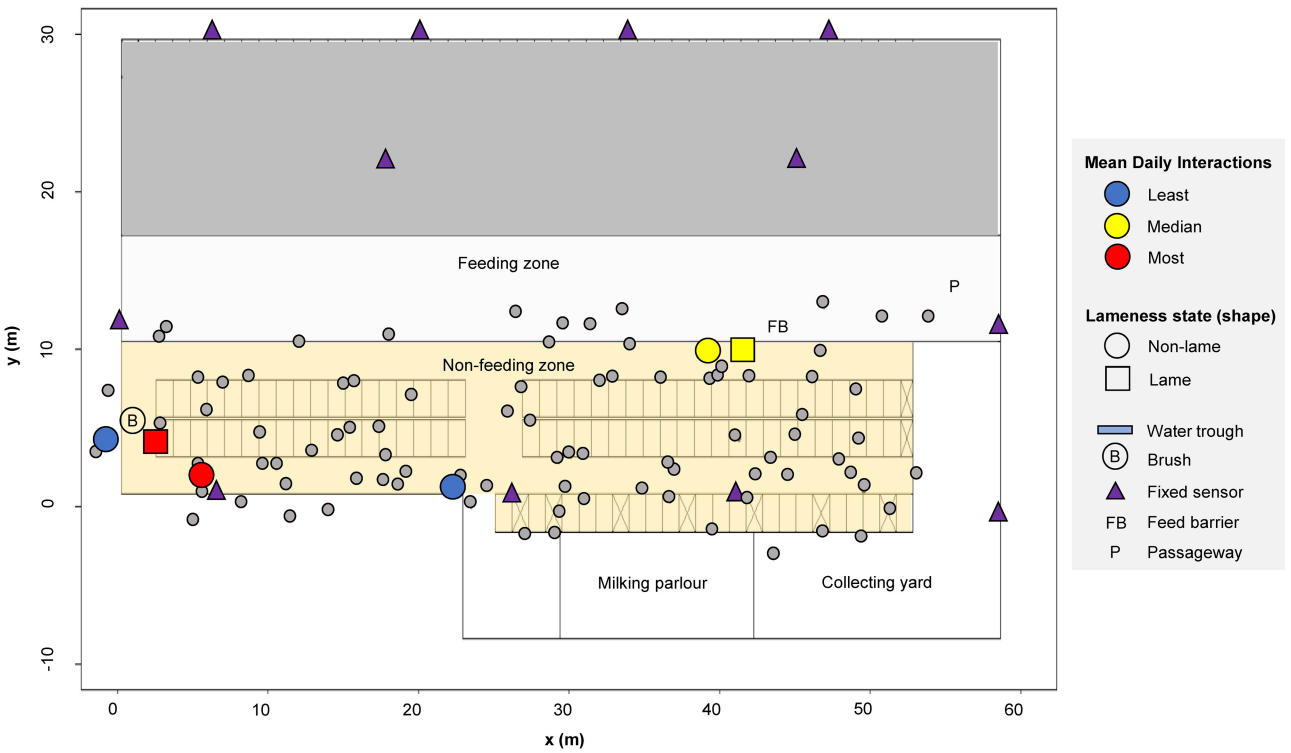


**FIGURE 1 |** Barn layout showing ($n = 92$) example cow locations recorded on 01/10/2020 at 05:09:50. A highlighted illustrative subset of cows ($n = 6$) are colored according to their mean daily interactions: least = blue (cow ID = 3124 and 3317), median = yellow (cow ID = 3602 and 3132), most = red (cow ID = 635 and 3361). Data for each individual cow is indicated with a small circle. The area shown in gray (upper barn area) was not used by this group of cows during the study period.

passage. Cows were milked three times per day (morning, 5 a.m.; afternoon, 1 p.m.; and evening, 9 p.m.) and provided with a total mixed ration once daily during morning milking; fresh feed was pushed up several times throughout the day. Health status, parity and days in milk were downloaded from the farm records, held in UNIFORM- (UNIFORM-Agri, Somerset, UK). A specific study of the effects of lameness on behavior with a smaller subset of the same herd group, within the same barn environment but over a different time period, has previously been reported (53, 57).

During the study, cows were assigned a mobility score fortnightly as they exited the parlor (on the 30/9/2014, 13/10/2014, and 27/10/2014), using the AHDB mobility score (51). A mobility score of 0- 3 was assigned, where 0 is good mobility, 1 is imperfect mobility, 2 is impaired mobility and 3 is severely impaired mobility (**Supplementary Material 1**). If a score was not recorded, "NS" was noted. For this study, cows with score 2 or 3 were considered as clinically lame (L) and cows with scores 0 or 1 were considered non-lame (NL). Cows scored as not lame for two successive scoring sessions (NL-NL-L or L-NL-NL) were classed as "dominant not lame," and cows scored as "dominant lame" for most sessions (L-L-NL or NL-L-L) were classed as lame. Cows that changed status twice within the study (NL-L-NL or L-NL-L) or those with missing data were not included in the lameness classification. For the purposes of the main analysis presented here, we combine "lame" and "dominant lame" cows into a single group ("lame"), and similarly "non-lame" and "dominant non-lame" cows are combined into a single group ("non-lame"). In total, 48 of the 92 cows within the study group were classified as either "lame" (22 cows) or "non-lame" (26 cows) using this approach (**Supplementary Material 1**), and were included in the part of our analysis focusing on lameness differences. Our results are qualitatively similar if we do not combine the groups and keep four separate classifications for lameness, see **Supplementary Material 1**.

## Local Positioning System

Cows were each fitted with a mobile Oms500 (Omnisense Ltd, Cambridge, UK) combined local-positioning and accelerometer sensor, attached to a weighted neck collar to ensure the sensors remained stable in the same orientation. The sensors deployed on the cows form a localized wireless network that uses triangulated radio signal communication to automatically determine the relative local position of every cow in the herd, at a temporal resolution of 0.1 Hz throughout the full study duration. Additional fixed sensors were strategically positioned throughout the barn to fix the absolute spatial location of each sensor and to maximize the sensor network performance (**Figure 1**). The performance of this specific sensor system in the same barn environment was evaluated in (53), who reported a 50% circular error of probability (CEP) measurement of 1.07 m for a static sensor (not mounted on a cow) and 1.90 m for a sensor mounted

on a standing cow (i.e., 50% of all measurements lay within 1.07 m of the mean location of static sensors and within 1.90 m of the mean location of cow mounted sensors). In the same study, mean distance errors of 2.66 m (static sensors) and 2.80 m (sensors on standing cows) were also reported.

## Pre-processing and Cleaning of Positional Data

All data processing and analysis was conducted in R for Windows 3.6.3 64 bit, with RStudio (68, 69). Extended interruption occurred because of a system malfunction on three of the study days (09/10/2014, 27/10/2014, and 31/10/2014); these incomplete days were not included in the analysis. Data cleaning and analysis were conducted on the 92 cows which were continuously present in the free-stall barn throughout the study duration ($d = 28$), see **Supplementary Material 2** for full details. In the first pre-processing step, location data further than a 3 m buffer distance outside the main barn area were removed; the 3 m buffer was included to avoid excluding data due to minor positional inaccuracies. Data removed at this stage included (correct) locations recorded in the milking parlor and collecting yard (where the cows were constrained for up to 3–4 h per day in total during the three milking events), as well as (incorrect) erroneous locations entirely outside the barn area. In total 22.81% of the original data was removed in this step. An automated "cleaning" algorithm was then used to identify and remove any nonsensical positional data (e.g., sensors apparently getting "stuck" in exactly the same, or a similar, point location for several consecutive time points, often shortly after the system reset at the end of each day; 3.06% of original data removed). The remaining location data were smoothed to remove noise using a simple moving average with a window size of 15 time points (corresponding to 150 s; 0.17% of original data removed due to losing 7 points at the start and end of the time series because of the smoothing window). A final combination of automated cleaning, and manual observation and checking, were then used to remove any further nonsensical data identified (e.g., cows that stayed relatively stationary for most of an entire day; 0.01% of original data removed). In total, 26.05 % (5,675,319 points) of the total original data points were removed through these pre-processing and data cleaning stages (see **Supplementary Material 2**); a total of 16,114,423 data points remained for the subsequent analysis.

## Protocol for Determining Proximity Interactions

Using the smoothed and cleaned positional data, an interaction was defined between dyads (each pair of cows) using a protocol based on sustained proximity (radial metric distance) over a specified time period, and was hence non-directed (if cow A is close to cow B, then B is close to A, and so on). In **Supplementary Material 3** we explain how and why we selected a "strict" protocol for identifying proximity interactions. The protocol specifies that, for a given dyad, all inter-cow distances over a time period of $t = 60$ s (i.e., 6 time points at 0.1 Hz) must be contained within a radius of $r = 3$ m for an interaction to be identified. While this parameter choice is consistent with previous studies (e.g., 14,16), we also considered a range of other parameter values for $r$ and $t$, as well as less stringent protocols (where only a certain percentage of points within the specified time period need to be within the radius for an interaction to be identified). Using observed data of ($n = 35$) known proximity interactions we were able to validate our algorithm and determine the sensitivity (true positive rate) of this protocol (0.83); it was not possible to estimate the specificity using this observed data, but the $r$ and $t$ parameters were chosen to reduce the expected false positive rate, as well as taking into account practical and biological considerations, including the sensor mean error distance and the typical size of a dairy cow (see **Supplementary Material 3** for details). It should also be noted that qualitatively similar results were obtained when using $t = 40$, 80, 100 s (for $r = 3$ m) (**Supplementary Material 3 Tables 6–8**) and $r = 1$, 2, 4 and 5 m (for $t = 60$ s) (**Supplementary Material 3 Tables 2–5**), and hence our conclusions should be robust to this parameter choice.

Positional data within the barn were filtered by coordinate into functional zones: the "feeding zone" (defined as the feeding passage and nearest passageway; see $10.5\,\text{m} \leq y \leq 17.2\,\text{m}$ in **Figure 1**), the "non-feeding zone" (cubicles and passageways; $1.62\,\text{m} \leq y \leq 10.5\,\text{m}, -1.6\,\text{m} \leq x \leq 58.6\,\text{m}$ in **Figure 1**) and the "full barn" (the combined feeding and non-feeding areas); a buffer of 3 m was used around each zone. The proximity protocol defining an interaction described above was subsequently applied to the data for every given dyad located in each functional zone, outputting the total number of interactions over the course of each day. A non-directed weighted matrix for every given day ($d = 28$) and functional zone was produced, holding the number of interactions recorded for every possible dyad (92 x 92). The matrices were therefore symmetrical, with "NA" inputted along the diagonals of each.

## Network Visualization

The interaction matrices for each day, for the full barn, and each functional zone, were converted into network graphs, using the package "igraph" (70) in R (68, 69), where nodes represent individuals ($n = 92$), and edges represent interactions between dyads, with increasing weight (more interactions) indicated by increasing width of the edges. The Fruchterman-Reingold layout algorithm was used to determine the node positions; connected nodes are pulled toward each other and unconnected vertices are repelled.

## Social Network Analysis

The edge density, the proportion of direct ties in a network relative to the total ties possible, was calculated for the full barn and functional zones (feeding and non-feeding zone). Cows periodically entered and left the feeding zone, so edge density was expected to be lower in this zone, in comparison to the non-feeding zone. The networks were also assessed for components, to reveal any potential divisions or isolated individuals, which could be linked to social assortment by lameness (or other factors) in later analysis.

Permutations are used to test the normality of observed network data and are essentially a form of null model (71, 72).

A widely used method to account for the non-independence of dyads in SNA is by using a node-level permutation (71, 72). Node identities are randomized, and the original test statistic is compared against permuted test statistics. Here we implement node-level permutations to test our hypotheses by randomizing the identity of cows ($q = 10{,}000$ in equation 1). A test statistic, comparing a given measure, i.e., differences in daily interactions between lameness states etc., was calculated for each permutation ($t_p$). If the proportion of permuted test statistics was equal to or more than the original test statistic ($t_o$), was $\geq$ 5% ($p \geq 0.05$) (see equation 1), then the null hypothesis was accepted i.e., there was no significant difference in the measure between the groups. A Bonferroni correction was applied to the $p$-value to account for multiple comparisons on the same dataset. As computing an exact $p$-value is not possible with a finite number of permutations, if the $p$-value was calculated to be zero a biased estimator was applied: one was added to both the numerator and denominator of Equation 1, following the suggestion in (73).

$$p = \frac{\Sigma\left(t_p \geq t_o\right)}{q} \quad (1)$$

## Social Differentiation

As the data on daily interactions was found to be not normally distributed (Shaprio-Wilk normality test; W = 1.00, 1.00, 0.98, $p = 0.04$, $< 0.01$ and $< 0.001$, for the full barn, feeding zone and non-feeding zone, respectively), a Kruskal-Wallis Rank Sum test was conducted to assess whether there is a significant difference in the median daily interactions individuals had, with 10,000 node-level permutations to account for non-independence of dyads.

The interactions between each dyad may be uniformly distributed across an interaction matrix for a given day, or specific dyads may interact more or less than other dyads. The structure of a network can be assessed by comparing the number of observed interactions between every given dyad with the number of expected interactions between every dyad. To assess whether associations between individuals were more heterogeneous than we would expect given a null hypothesis that all dyads associate uniformly, the following statistic for social differentiation (S) was calculated (see Equation 2) based on (29), Appendix 9.4, and following (14):

$$S = \frac{\sum_i^n \sum_j^n (O_{ij} - E_{ij})^2}{n(n-1)} \quad (2)$$

As shown in Equation (2), the difference between the observed number of interactions and the expected number of interactions was summed for each dyad, and then divided by the total number of dyads ($n = 4186$ [= ((92 x 91)/2)]), for each day.

## Temporal Variation in Sociality

A Kruskal-Wallis Rank Sum test was conducted to assess whether there was a significant difference in median daily interactions between days, for each functional zone, with 10,000 permutations. Pearson's correlation was used to test if temporal variations in daily interactions were correlated across time in each functional zone, and then with mean daily temperature.

To assess whether the network structure was stable or varied over time, seven interaction matrices were created, each holding the average number of interactions between dyads ($n = 4186$) over four consecutive days. Each consecutive network was compared by conducting a Mantel Test (8, 74). The "mantel" function was used, from the "vegan" package in R (75). As the interaction data within the matrices were not normally distributed (as shown through a one-sample Kolmogorov-Smirnov test), a Spearman's Rank Sum test was used to calculate a Mantel statistic $Z$, for each consecutive averaged matrix, with 10,000 permutations and Bonferroni correction applied to account for multiple comparisons. We also completed a similar analysis using shorter- and longer-day partitions, and results were found to be qualitatively similar (**Supplementary Material 3 Table 9**).

## Impact of Lameness Status, Parity, and Days in Milk on Sociality

### *Lameness Status*

The mean daily interactions between non-lame ($n = 26$) and lame ($n = 22$) cows were compared using a two-tailed Wilcoxon test, with 10,000 permutations

Node degree (the number of immediate neighbors each node in the network has) was compared between non-lame and lame cows. As a cumulative measure, node degree is less prone to sampling error, such as temporal loss of signal of the sensor system, than other measures such as betweenness (the number of shortest paths that pass through a given node), which can change dramatically with removed or missing data (76), so mean node degree was compared between non-lame and lame cows. Local clustering coefficient (the extent to which nodes cluster in a graph, calculated by the proportion of connections a node has with its neighboring nodes divided by the maximum number of connections that could exist in this neighborhood) was also compared between non-lame and lame cows. The mean node-level measures, calculated for each individual over the full study period, were compared between lameness states using two-tailed Wilcoxon tests with 10,000 permutations (Shapiro-Wilk normality test, $p < 0.01$).

A matrix was created, showing the absolute differences in lameness between all dyads ($n = 1128$), as in (16) (e.g., if cow A was lame, a score of 1 was assigned, and cow B was not lame, a score of 0 was assigned, and their absolute difference would be 1). The absolute difference matrix was compared to the original interaction matrix for every given day, using a Mantel test again with Spearman's Rank Correlation Coefficient. Bonferroni correction was applied to account for multiple comparisons ($n = 28$).

### *Parity and Days in Milk*

To assess whether parity and days in milk (DIM) affected social assortment, a matrix was created, showing the absolute differences in parity between all dyads ($n = 4186$), as in (16) (e.g., if cow A had a parity of 1, and cow B had a parity of 3, their absolute difference would be 2). An absolute difference matrix

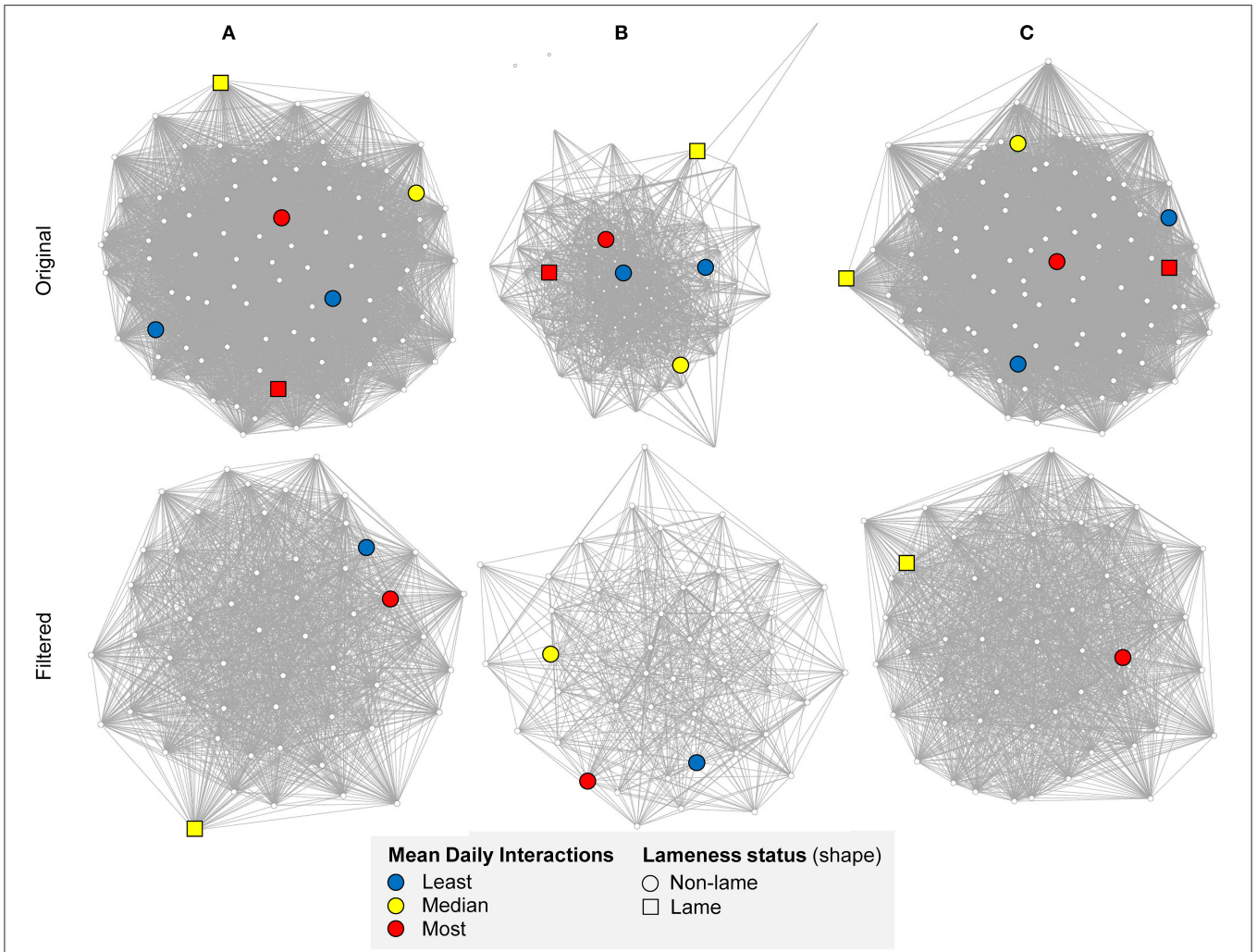


**FIGURE 2 |** Undirected original and filtered (by mean degree) networks on a randomly chosen day, 01/10/2014, in **(A)** the full barn, **(B)** feeding zone, and **(C)** non-feeding zone, showing mean daily interactions between cows ($n = 92$ in original networks). Thicker edges indicate a higher number of daily interactions. The Fruchterman-Reingold layout algorithm was used to determine the node positions; unconnected vertices are repelled. The highlighted illustrative subset of cows correspond, respectively to the least (blue, cow ID = 3124 and 3317), median (yellow, cow ID = 3132 and 635), and most (red, cow ID = 2273 and 2266) mean daily interactions, with squared nodes representing lame cows. A clearer network structure is shown after filtering, with a more uniform distribution of interactions in the main barn and the non-feeding zone in comparison to the feeding zone. Created in RStudio using the "vegan" package (68, 69, 75).

for days in milk (DIM) was also created. The absolute difference matrix for a given attribute was compared to the original matrix for every given day, using a Mantel test (as described in Section Lameness Status).

# RESULTS

## Basic Network Measures and Visualization

**Figure 2** compares visualizations of the original and mean node degree filtered networks for the full barn, and the feeding and non-feeding zones. A key notable difference between the networks is that the full barn network was more connected than the non-feeding zone network (0.02 difference in edge density) and the feeding zone network (0.63 difference in edge density; **Figure 1**; **Table 1**). This is expected since interactions occurring at the boundaries of the feeding and non-feeding zones were likely to be missed when considering these zones separately. The non-feeding zone network was more connected than the feeding zone network (0.31 difference in edge density) (**Figure 2**; **Table 1**).

The full barn and non-feeding zone networks remained as one component, whereas in the feeding zone network one to three individuals isolated from the main component on each day (**Table 1**).

## Inter-individual Variation

Throughout the following analysis and presentation of results, a subset of individuals at the middle and extreme ends of the data set are highlighted to aid interpretation and to illustrate the extent of the observed data: two with the lowest mean daily interactions

**TABLE 1 |** Overview of results using a spatial threshold radius of $r = 3$ m and time duration of $t = 60$ s to define an interaction for the full barn (FB) and the functional zones: feeding zone (FZ) and non-feeding zone (NFZ): basic network measures (original and filtered by mean degree), inter-individual variation, temporal variation in sociality, lameness status, and parity and days in milk, where (M)DI = (median) daily interactions.

| | Measure | Test value (*p*-value) | | | Summary |
|---|---|---|---|---|---|
| | | Full barn | Feeding zone | Non-feeding zone | |
| Basic network measures | Mean edge density ($d = 28$) | 0.96 | 0.33 | 0.94 | The networks are highly dense, more so the NFZ than the FZ. |
| | Components (by day) ($d = 28$) | 1 | 2-6 | 1 | The networks typically consist of one component. |
| Inter-individual variation | Inter-individual differences in median DI ($n = 92$) | K-W = 26.53 (**$p < 0.001$**) | K-W = 851.71 ($p = 1$) | K-W = 19.21 (**$p < 0.001$**) | **Inter-individual variation in DI in the NFZ** but not in the FZ or the FB. |
| | Social differentiation (SD) ($n = 92$) | SD between ≤ 92.96 % of dyads (**$p < 0.01$**) | SD between 100 % of dyads (**$p < 0.01$**) | SD between 92.96 % of dyads (**$p < 0.01$**) | **Social differentiation present in all networks.** |
| Temporal variation in sociality | Difference in median DI between days ($n = 92$, $d = 28$) | K-W = 2252.30 ($p = 1$) | K-W = 61.00 ($p = 1$) | K-W = 2268.9 ($p = 1$) | No difference in DI between days in all networks. |
| | Relationship between MDI and days ($n = 92$, $d = 28$) | Pearson correlation, $\rho = 0.03$ ($p = 0.88$) | Pearson correlation, $\rho = 0.55$ (**$p < 0.01$**) | Pearson correlation, $\rho = 0.02$ ($p = 0.90$) | MDI correlated over time in the feeding zone but not in the non-feeding zone. |
| | Relationship between MDI and temperature ($n = 92$, $d = 28$) | Pearson correlation, $\rho = 0.04$ ($p = 0.83$) | Pearson correlation, $\rho = -0.09$ ($p = 0.66$) | Pearson correlation, $\rho = 0.04$ ($p = 0.82$) | Weak correlation between MDI and temperature in both functional zones. |
| | Relationship between four-day block consecutive networks (six networks, $n = 92$ per network) | Mantel test, range of $R_s = 0.03$ to 0.23 (**$p \leq 0.001$**) for three comparisons (day blocks 1–2, 2–3, 5–6); range of $R_s = -0.04$ to −0.001 ($p > 0.23$) for three comparisons (day blocks 3–4, 4–5, 6–7) | Mantel test, range of $R_s = 0.20$ to 0.31 (**$p < 0.001$**) | Mantel test, range of $R_s = 0.05$ to 0.24 (**$p < 0.01$**) for four comparisons (day blocks 1–2, 2–3, 5–6, 6–7); range of $R_s = -0.04$ to 0.01 ($p = 1$) for two comparisons (day blocks 3–4, 4–5) | Weak correlation between all consecutive networks. |
| Individual characteristics | Difference in mean DI between non-lame ($n = 26$) and lame cows ($n = 22$) | Wilcoxon test, W = 297 ($p = 0.56$) | Wilcoxon test, W = 342 ($p = 0.86$) | Wilcoxon test, W = 276 ($p = 0.40$) | No difference in DI between non-lame and lame cows in both functional zones. |
| | Difference in mean clustering coefficient between non-lame ($n = 26$) and lame cows ($n = 22$) | Wilcoxon test, W = 392 ($p = 0.98$) | Wilcoxon test, W = 284 ($p = 0.53$) | Wilcoxon test, W = 398 ($p = 0.99$) | No difference in clustering coefficient between non-lame and lame cows in either functional zone. |
| | Difference in mean node degree between non-lame ($n = 26$) and lame cows ($n = 22$) | Wilcoxon test, W = 304.5 ($p = 0.63$) | Wilcoxon test, W = 321.5 ($p = 0.25$) | Wilcoxon test, W = 241.5 ($p = 0.17$) | No difference in node degree between non-lame and lame cows in either functional zone. |
| | Social assortment by lameness status by day, $n = 48$) | Mantel test, $R_s = 0.11$ (**$p < 0.01$**) for day 16; range of $R_s = -0.07$ to 0.05 (**$p = 1$**) for remaining 27 days | Mantel test, range of $R_s = -0.06$ to 0.04 ($p = 1$ for all days) | Mantel test, range of $R_s = -0.07$ to 0.06 ($p > 0.80$) | Cows did not socially assort according to their lameness status, parity, or DIM in either functional zone. |
| | Social assortment by parity (by day, $n = 92$) | Mantel test, range of $R_s = -0.02$ to 0.03 ($p = 1$ for all days) | Mantel test, range of $R_s = -0.05$ to 0.03 ($p = 1$ for all days) | Mantel test, range of $R_s = -0.02$ to 0.03 ($p = 1$ for all days) | |
| | Social assortment by DIM (by day, $n = 92$) | Mantel test, range of $R_s = -0.03$ to 0.03 ($p = 0.80$ for all days) | Mantel test, range of $R_s = -0.03$ to 0.04 ($p = 1$ for all days) | Mantel test, range of $R_s = -0.03$ to 0.03 ($p > 0.44$ for all days) | |

*Significant results ($p < 0.05$) are in bold.*

over the full study period (cow ID = 3324 and 3317 with mean daily interactions of 1955 and 1956, respectively), two with mean daily interactions closest to the median (cow ID = 2602 and 3132, with mean daily interactions of 2084 and 2085, respectively), and two with the highest mean daily interactions (cow ID = 635 and 3361, with mean daily interactions of 2266 and 2273,

respectively); across the full herd the mean daily interactions were 2093 (median = 2085, standard deviation = 76.63).

There was significant inter-individual variation in daily interactions in the non-feeding zone (Kruskal-Wallis chi-squared [hereafter K-W] = 19.21, df = 91, after 10,000 permutations, $p < 0.01$), but not in the full barn (K-W = 26.53, df = 91, after 10,000 permutations, $p < 0.001$) or the feeding zone (K-W = 851.71, df = 91, after 10,000 permutations, $p = 1$).

**Figure 3** illustrates the lack of inter-individual variation in daily interactions in the full barn and the non-feeding zone, and the greater inter-individual variation in daily interactions in the feeding zone for the highlighted subset of individuals.

Social differentiation was observed across the full barn (> 92.96 % of dyads, p < 0.01 across days), the feeding zone (100 % of dyads, $p < 0.01$ across days), and the non-feeding zone (92.96 % of dyads, $p < 0.01$ across days), see **Table 1**.

## Temporal Variation in Sociality

There was no significant difference in median daily interactions between days in the full barn (K-W chi-squared = 2252.30, df = 27, after 10,000 permutations, $p = 1$; **Table 1**), feeding zone (K-W = 61.00, df = 27, after 10,000 permutations, $p = 1$; **Table 1**), nor in the non-feeding zone (K-W = 2268.9, df = 27, respectively after 10,000 permutations, $p = 1$; **Table 1**).

**Figure 4** highlights the temporal instability in both the functional zone networks. Although there were no clear trends over time, where there were changes these are seen to be highly correlated across all individuals in the feeding zone ($n = 92$; Pearson's coefficient [hereafter $\rho$] = 0.02, $n = 92$, $p = 0.90$) (**Figure 4**). Conversely, individual interactions in the feeding zone showed much more random variation than in the non-feeding zone ($\rho = 0.55$, $n = 92$, $p < 0.01$), as demonstrated with the highlighted subset of individuals (**Figure 4**). There was a weak but non-significant relationship between mean temperature and mean daily interactions across days in both the feeding zone ($\rho = -0.09$, df = 26, $p = 0.66$; **Table 1**; **Figure 4**) and non-feeding zone ($\rho = 0.04$, df = 26, $p = 0.82$; **Table 1**; **Figure 4**).

In the feeding zone, there were significant weak positive correlations between all the four-day block averaged- consecutive networks ($n = 7$, comparisons = 6) (range of Spearman's coefficient [hereafter $R_s$] across days = 0.20 to 0.31, after 10,000 permutations and Bonferroni correction, $p < 0.001$ for all comparisons; **Table 1**; **Figure 5**). In the full barn there were also weak correlations between the four-day averaged consecutive networks (range of $R_s = 0.03$ to 0.23, after 10,000 permutations and Bonferroni correction, $\boldsymbol{p \leq 0.001}$ for three comparisons (day blocks 1–2, 2–3, 5–6); range of $R_s$ −0.04 to −0.001, after 10,000 permutations and Bonferroni correction, $p > 0.23$ for three comparisons (day blocks 3–4, 4–5, 6–7). In the non-feeding zone, there were inconsistent weak correlations between consecutive networks (range of $R_s = 0.05$ to 0.24, after 10,000 permutations and Bonferroni correction, $\boldsymbol{p < 0.01}$ for four comparisons (day blocks 1–2, 2–3, 5–6, 6–7); range of $R_s = -0.04$ to 0.01, after 10,000 permutations and Bonferroni correction, $p = 1$ for two comparisons (day blocks 3–4, 4–5); **Table 1**; **Figure 5**). We also conducted this analysis using the original ($n = 28$), and two-, seven- and 14-day blocks, and we obtained qualitatively similar results (**Supplementary Material 3 Table 9**).

## Impact of Health Status, Parity, and Days in Milk on Sociality

### Lameness

Lame cows ($n = 22$) did not have significantly more mean daily interactions than non-lame cows ($n = 26$) in the feeding zone (Wilcoxon test statistic [hereafter W] = 342, $p = 0.86$ after 10,000 permutations; **Table 1**; **Figure 6**) nor in the non-feeding zone (W = 276, $p = 0.40$ after 10,000 permutations; **Table 1**; **Figure 6**).

In the feeding zone, lame cows did not show a significantly different mean clustering coefficient or degree than non-lame cows (W = 284 and 321.5, respectively, after 10,000 permutations, $p = 0.53$ and 0.25, respectively; **Table 1**; **Figure 6**). Similarly, in the non-feeding zone, mean clustering coefficient or degree did not differ between the lameness states (W = 398 and 241.5, after 10,000 permutations, $p = 0.99$ and 0.17 respectively; **Table 1**; **Figure 6**).

There was no significant social assortment by lameness in the feeding zone (range of across days $R_s = -0.06$ to 0.04), nor the non-feeding zone (range of across days $R_s = -0.07$ to 0.06) where, after Bonferroni Correction and 10,000 permutations, $p > 0.80$ in all cases for all days ($n = 28$; **Table 1**). In other words, cows with the same lameness state did not associate more than cows of different lameness states.

### Parity and Days in Milk

There was no significant social assortment by parity in the feeding zone (range of across days $R_s = -0.05$ to 0.03, after 10,000 permutations and Bonferroni Correction, $p = 1$ for all days; **Table 1**) or in the non-feeding zone network (range of $R_s$ across days = −0.02 to 0.03, after 10,000 permutations and Bonferroni Correction, $p = 1$ for all days; **Table 1**).

There is also no significant social assortment by DIM in the feeding zone (range of across days $R_s = -0.03$ to 0.04, after 10,000 permutations and Bonferroni Correction $p = 1$ for all days) or the non-feeding zone (range of $R_s$ across days = −0.3 to 0.03, after 10,000 permutations and Bonferroni Correction, $p \geq 0.44$ for all days; **Table 1**).

The results for social assortment by lameness, parity and DIM in the full barn network were similar to those of the non-feeding zone (results in **Table 1**).

# DISCUSSION

Within this study we found that the interaction network of the housed dairy herd was highly connected with significant social differentiation, interactions between cows were more heterogenous than expected by chance (18), but the network structure was temporally unstable. There was no evidence of preferential social assortment, showing cows did not associate more than expected by chance according to lameness state, parity, or days in milk (DIM).

Visualization of the full barn interaction network (**Figure 2**) illustrates that the herd was highly connected, as confirmed by the mean edge density (96%, **Table 1**). This indicates that each

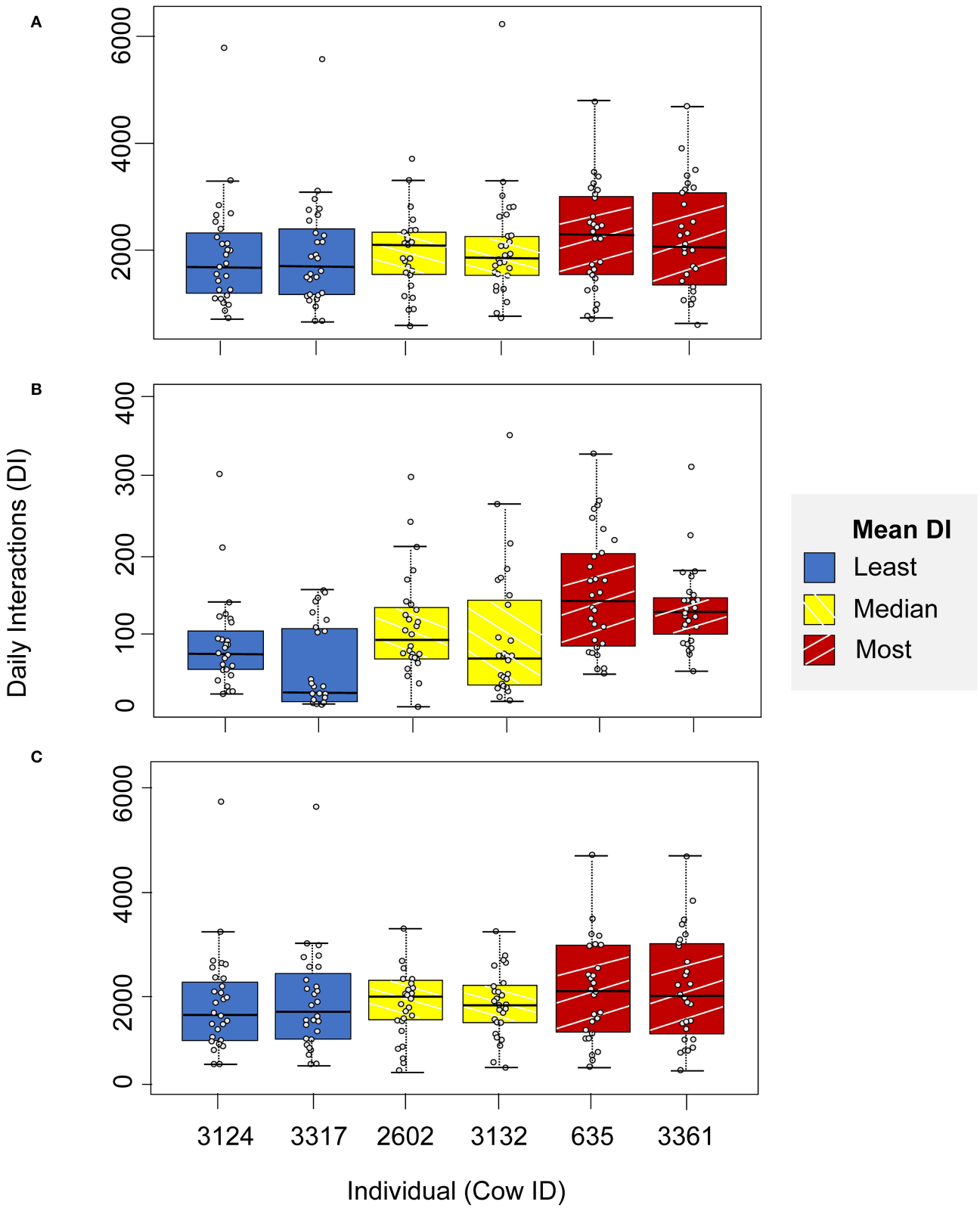


**FIGURE 3 |** Daily interactions in **(A)** the full barn, **(B)** feeding zone, and **(C)** non-feeding zone, for a highlighted illustrative subset of individuals: two individuals with the least mean daily interactions (blue, cow ID = 3124 and 3317), two with mean daily interactions closest to the median value (yellow, cow ID = 2602 and 3132) and two with the highest mean daily interactions (red, cow ID = 635 and 3361).

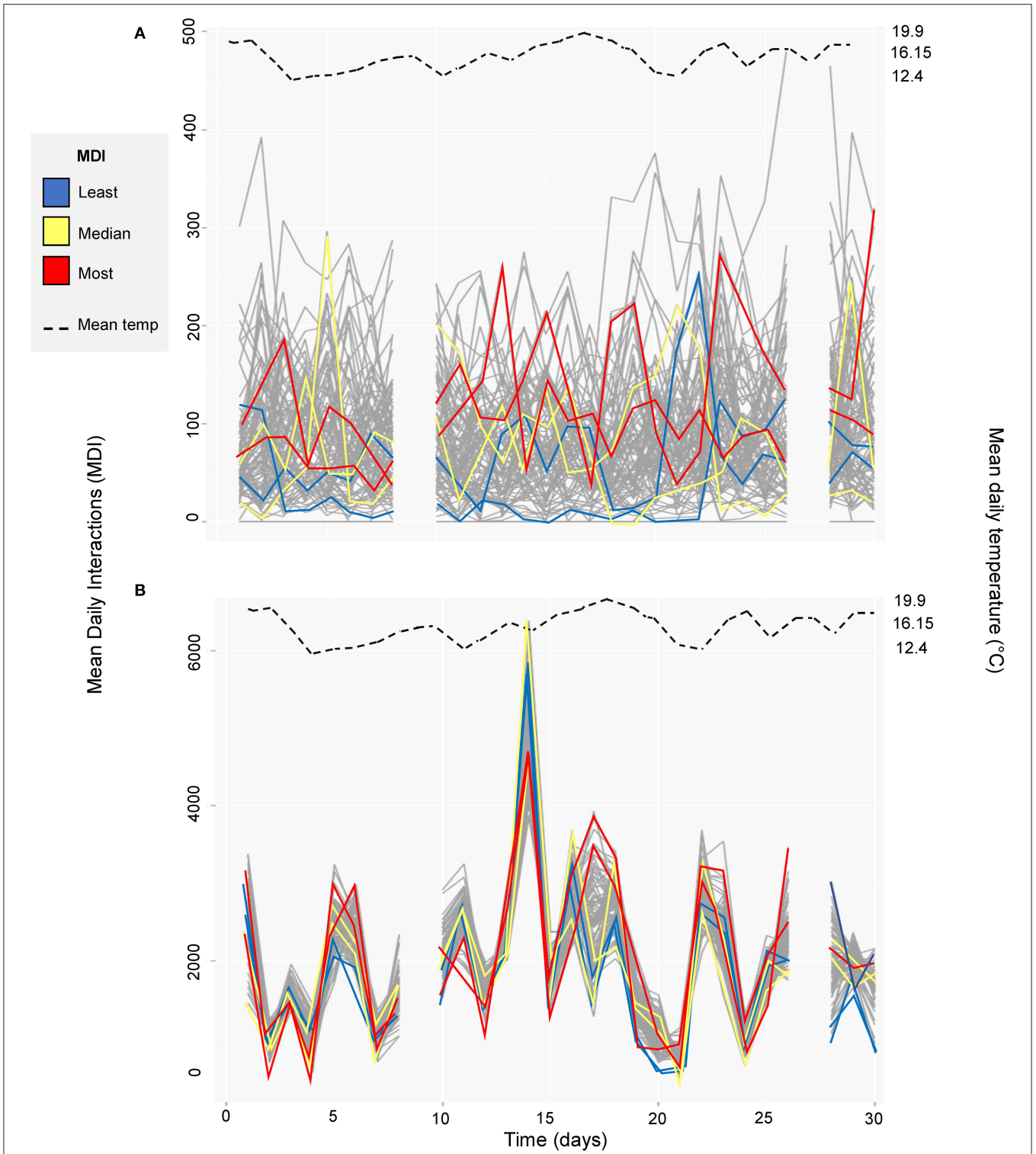


**FIGURE 4 |** Mean daily interactions across time (01/10/2014 to 31/10/2014 with days excluded from the study omitted) in **(A)** feeding zone, and **(B)** non-feeding zone. An illustrative subset of individuals are highlighted: two individuals with the least mean daily interactions (blue, cow ID = 3124 and 3317), two with mean daily interactions closest to the median value (yellow, cow ID = 2602 and 3132) and two with the highest mean daily interactions (red, cow ID = 635 and 3361). Data for each individual cow is indicated with a gray line. Mean daily temperature is shown with the dashed black line.

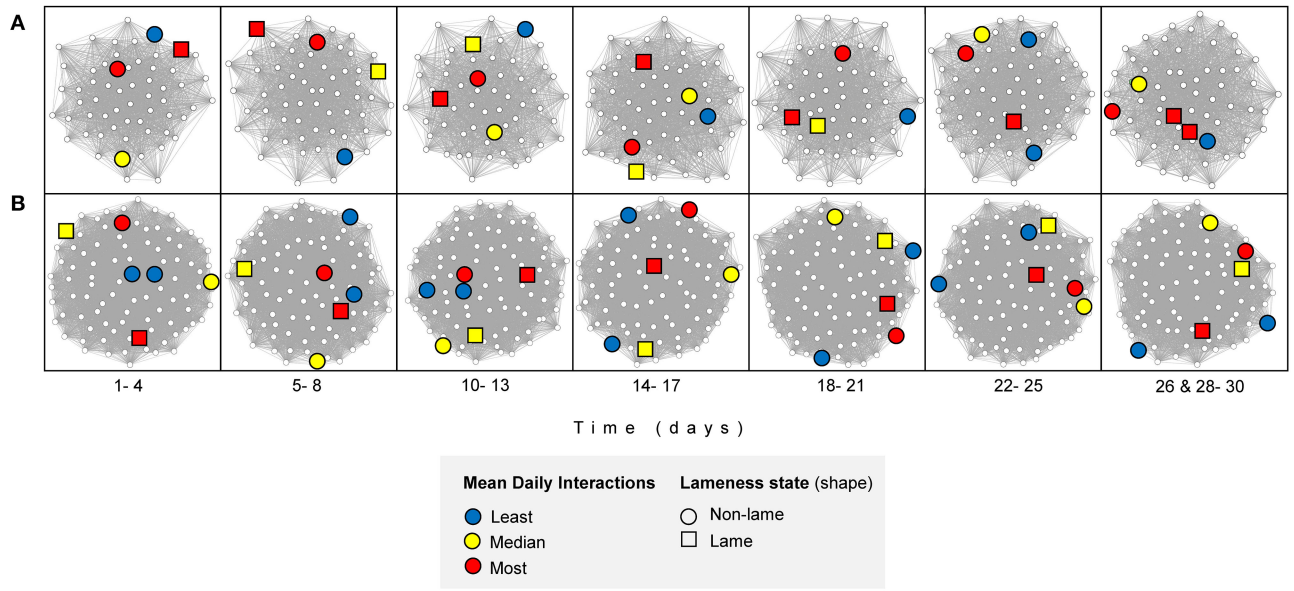


**FIGURE 5 |** Interaction networks, filtered by mean node degree, over time for **(A)** feeding zone, and **(B)** non-feeding zone. An illustrative subset of individuals are highlighted: two individuals with the least mean daily interactions (blue, cow ID = 3124 and 3317), two with mean daily interactions closest to the median value (yellow, cow ID = 2602 and 3132) and two with the highest mean daily interactions (red, cow ID = 635 and 3361). The Fruchterman-Reingold layout algorithm determined the node positions; unconnected vertices are repelled. Created in RStudio using the "vegan" package (68, 69, 75).

cow was likely to have had interactions with most other cows in the herd each day. It is not clear from this study whether these cows actively seek out and connect with their conspecifics, perhaps to maintain social structure in the group, or whether this high connectivity is a function of the building layout and high stocking density. It must be acknowledged that, due to building works on the farm, the stocking rates were high during our study period (feed space = 0.48 m per cow, lying space = 0.72 cubicles per cow). This may have reduced the ability of the cows to actively choose with whom to be in close proximity with. In agreement with this study, high connectivity was also reported for cows housed in loose straw yards with concrete loafing areas with moderate (to high) stocking rates of 9.50 $m^2$ per cow to (7.66 $m^2$ per cow) from sensor derived proximity measurements (14, 15). Lower edge densities have been reported in a grazing system, in (7), but in their study an interaction was based on the occurrence of specific behaviors considered to increase the risk of disease transmission rather than social proximity. Lower edge density and a sparse structure was also reported for cows housed in cubicles at a moderate stocking rate (1.03 cubicles per cow), but the group in their study only comprised of 36 cows and interactions were only recorded during two 15 min time slots per day, therefore not capturing changes in location and near neighbors throughout the day (77). Further investigations of dairy cows in a range of housing types and stocking rates are needed to determine if cows are naturally highly connected or whether aspects of the commercial dairy lead to cows spending time in proximity to a greater number of conspecifics.

Analysis on the interaction networks revealed significant inter-individual variation in daily interactions in the non-feeding zone, but not across the feeding zone or when considering the full barn (**Figure 3**; **Table 1**). The feeding zone is likely to be a more dynamic location than the loafing and resting areas. Feed bouts are shorter than lying bouts and cows will begin and end their eating bouts at different times, leading to a greater turnaround of contacts at the feed face than other areas of the barn. It is possible however, that cows have greater control over the individual interactions they have in the non-feeding zone and therefore we are able to observe a greater degree of individuality. Researchers have demonstrated that inter-individual variation in sociality is an individual trait in dairy cows (28) influenced by dominance status and personality traits. This may affect an individual's ability to gain resources, such as cubicles, impacting their proximity interactions in the non-feeding zone (17, 21), as also speculated by (14), although we cannot distinguish between these potential factors in this study.

The structure of the interaction network was weakly correlated over time (**Figures 3**, **4**), and individuals periodically isolated from the main network component of the feeding zone (**Table 1**). These individuals were not the same each day, and they were not of the same lameness status, suggesting their isolation was due to them choosing not to feed at the same time, or being unable to compete due to the lack of space. The overall herd was subject to changes throughout the study period, with the addition and removal of cows outside of the study group ($n = 92$, whole herd = 100–111 cows on a given day), which could have affected the social structure of the herd. In (28), while introductions of new cows to a stable group did not affect the sociality of individual cows, it did weaken the overall social network. The highly connected network in (14) was also subject to changing

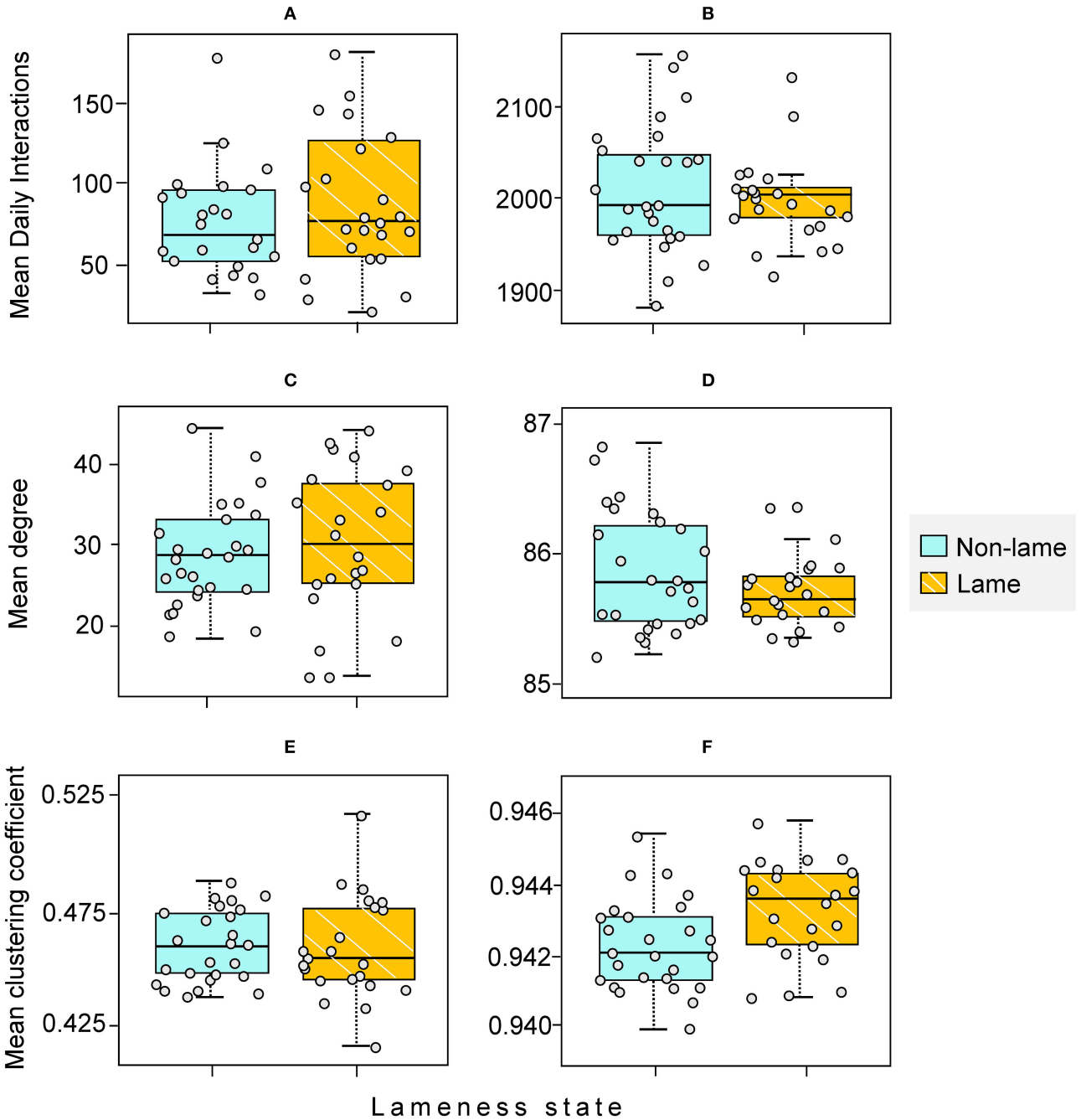


**FIGURE 6 |** Comparison of Mean Daily Interactions and node-level measures (degree and clustering coefficient) between non-lame (NL) ($n = 26$) and lame (L) ($n = 22$) cows. **(A,C,E)** feeding zone; **(B,D,F)** non-feeding zone. The horizontal line in each boxplot represents the median value. The mean [standard deviation] values for NL and L cows are given, respectively by: **(A)** 74 [35] and 90 [44]; **(B)** 2015 [70] and 2006 [45]; **(C)** 27.92 [8.53] and 30.14 [9.37]; **(D)** 85.85 [0.45] and 85.68 [0.26]; **(E)** 0.44 [0.09] and 0.46 [0.02]; **(F)** 0.94 [0.001] and 0.94 [0.001]. Each individual cow is indicated with a small circle.

group composition and the researchers similarly reported weak to moderate correlations in structure between consecutive one-week networks. Further analysis on the temporal stability of dairy cow networks whilst removing specific individuals could aid management.

There were no significant correlations between daily interactions and temperature in this study (**Figure 4**, **Table 1**). However, the study period was selected based on there being a relatively stable temperature throughout with temperature low enough not to induce heat stress. Cows have been shown

to modify their collective behavior, in terms of clustering for example, or individual behaviors in extreme heat conditions, or show long-term signs of heat stress due to high stocking densities (78–81). Therefore, environmental temperature and even individual cow temperature should be considered when monitoring the herd social structure over longer study periods. Furthermore, the social network may have been more dynamic than initially envisioned due to factors not accounted for, such as farm management actions or treatment interventions (82).

Considering the known social withdrawal response of unhealthy cows (83), it might be predicted that lame cows would be less willing to compete for preferred food or access to cubicles, but no differences in the sociality or positioning were found between lame and non-lame cows (**Figure 6**, **Table 1**). At a particularly high stocking rate in intensive cubicle housing, there may have been little opportunity for the 22 "lame" cows identified in this study to self-isolate. Lame cows have been shown to modify their space-use in this barn, but this was with access to an additional loafing area at the end of the cubicle shed which would make social distancing easier than in this study (57). Furthermore, (84) found that lame cows received approximately twice as much allogrooming as cows that were non-lame, and this explanation would also support our finding of no individual-level social assortment by lameness state i.e., cows of the same lameness state did not associate more or less than expected (84) (**Table 1**). When interpreting the result above we should consider that use of a visual locomotion score is not without the potential for classification errors, especially when scoring large groups of cows at the parlor exit as was the case in this study. It has been reported that mild claw lesions are not always accompanied with a corresponding increase in locomotion score, indicating that locomotion scoring even by trained observers may not be sensitive enough to detect all lameness cases (35). Indeed in a previous study a predictive statistical model correctly classified two cows that were incorrectly classified by observer locomotion scoring (57). Cows with dominant lameness status were also discretely grouped as either "non-lame" or "lame" during analysis (38, 85) (**Supplementary Material 1**), and these cows may have behaved differently during various time periods of the study. Nonetheless, this study demonstrates a potential way to assess the influence of health status on social interactions within a typical herd. Quantitative measures of individual social interactions and network position may be useful indicators to use within automated monitoring approaches in PLF.

Social differentiation was present in both functional zones (**Table 1**); some dyads interacted more than others, as similarly shown in (15, 86). A number of previous studies have indicated social differentiation can occur with age, as cows of a similar age would have had greater opportunity to develop social ties with one another (86, 87), particularly if they calved at similar times. In addition, stronger bonds may also form between calves born at similar times, who remain together throughout rearing before joining the milking herd; cows have been shown to invest more time and energy into relationships with herd members sharing long-term experiences (88). Our study does not find that cows differentiate by parity, a proxy for age. While parity may give an indication as to a cow's experience in the herd and may contribute to her personality traits, this measure is probably too coarse to identify cows with historical associations, such as shared calf cohorts, which has been suggested to result in stronger bonds. In this study a recent shared transition period, as indicated by similar DIM, was not sufficient to result in differentiation on this basis. This is in line with the findings of (89), where recent familiarity with cows had no effect on lying down behaviors of cows transitioning to the herd but early familiarity lead to greater synchrony of lying behaviors. Greater detail of the cohorts of cows kept from birth through to the milking herd, unmeasured in this study, may explain the social differentiation observed. It is possible that the high temporal variation of the network structure, and insufficient space within the barn may have impeded the ability to identify these structures. Alternatively, non-random associations may have been the result of cows of similar dominance rank positioning closely, with subordinates displaced from favorable feeding positions by dominant cows (20), particularly as feed space was limited to $< 0.60$ m/cow. Interactions may be more likely to develop between cows with similar energy requirements and motivation, and hence similar activity time budgets. For example, cows that spend more time eating may spend a lot of time near the feed face and hence position closely to similar cows (15, 86, 87). Stage of lactation affect the time an individual allocates to feeding, given that energy requirements vary with milk yield; for instance, dry matter intake is typically highest during mid-lactation (90).

When interpreting our results, it is important to consider potential limitations of the relatively novel technology and SNA techniques used in this study (82, 91). Although the proximity used to define an interaction was also tested for other radii and time durations, and similar qualitative results were obtained (**Supplementary Material 3**), any interactions detected were limited by the accuracy of the LPS system (2.66 m mean error for a static sensor). Additionally, a fundamental problem with this type of automated approach to identify proximity interactions is that we are unable to distinguish between which proximity interactions were true social interactions (e.g., allogrooming) and which were non-deliberate or non-social proximity events [e.g., due to the positioning of neighboring cows at the feed face (3, 82) or in cubicles (3)]. Our results are likely to contain both genuine sustained social interactions, as well as proximity events which were not directly social. Distinguishing between genuine social interactions and indirect or non-social proximity interactions is an open research question that requires further investigation. Our chosen proximity identification protocol was tested and validated using observational data and was found to have a sensitivity of 83% ($r = 3$ m and $t = 60$ s), but we were unable to directly estimate the rate of false positives and hence the specificity (**Supplementary Material 3**). Using a time duration of 60 s is likely to reduce the rate of false positives (compared to using a shorter time duration) but will also potentially exclude genuine social interactions of short duration. Multiple shorter interactions may be as socially relevant as longer sustained interactions. Our analysis was based on a comparison of daily-level network statistics and comparing these over time or between individuals with different lameness status, parity and DIM. It is quite plausible that, although the daily level behavior may be similar across the network, there could be significant individual variability in social interactions on a finer timescale (e.g., hourly

or less), particularly around key events such as feeding and milking, and this variability in social behavior may be linked to social status or health. A further limitation is that, although we included the vast majority of cows that were present in the herd throughout the study period ($n = 92$), there were cows that entered and left the group throughout this period, and hence some potential interactions involving these cows would not have been recorded. The effect of missing individuals on the conclusions drawn from a social network analysis are not well understood and this remains an open research question (82, 91). Despite the drawbacks to using proximity to detect potential social interactions, our approach based on using a local positioning system is useful for quickly accumulating the large datasets needed for SNA in an automated way (82).

## CONCLUSION

A local positioning sensor network was used to automatically monitor the spatial position of a large herd group of permanently housed dairy cows at high temporal resolution for a full month. Proximity interactions were identified by sustained periods of closeness between dyads. The proximity interaction network structure of the herd was highly connected, with significant differentiation in interactions between dyads, and high temporal variability. Lameness, parity, and days in milk were not found to directly influence social interactions or network position. This study demonstrates how automated sensor technology could be used to monitor the social structure of a large commercially relevant group of livestock, and how individual differences in social interactions and network measures could be used to potentially identify health differences between animals. Future work should aim to better distinguish social interactions from indirect non-social interactions and consider how interactions within a larger group may differ in different housing environments and at different stocking densities.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Royal Veterinary College Ethics and Welfare Committee under the unique reference number 2012 1223. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

EC, JA, DC, and NB contributed to the study design and secured grant funding. ZB and HH contributed to the study design and undertook data collection with assistance from NB. KC, JV, and EC undertook data analysis with assistance from DC. KC, TC, and EC prepared the manuscript. All authors reviewed and approved the final manuscript.

## FUNDING

This research was funded by the UK Biotechnology and Biological Sciences Research Council thorough grants BB/K002562/1 (EC and JV), BB/K002376/1 (JA and ZB), BB/K001302/1 (DC), BB/K003070/1 (NB). HH was supported by the Colin Spedding Memorial Research Studentship awarded by The Farm Animal Welfare Trust. KC is supported by an interdisciplinary faculty studentship from the University of Essex. The APC was funded by a UK Research and Innovation (UKRI) open access block grant awarded to the University of Essex.

## ACKNOWLEDGMENTS

We are very grateful to all the farm staff who helped to facilitate this study.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.583715/full#supplementary-material

**Data Sheet 1 |** Classification of lameness and additional related results. Further details of how individual mobility scores were used to classify lameness states: dominant lame (DL; $n = 10$), lame (L; $n = 12$), dominant non-lame (DNL; $n = 11$) and non-lame (NL; $n = 15$). Additional results are included where the former two groups were each compared to the latter two groups, in terms of mean daily interactions, node degree and clustering coefficient.

**Data Sheet 2 |** Data selection, cleaning, and processing. Further details and justification of the steps taken to select, clean and process the raw positional data collected during the study period. Only cows present throughout the entire duration of the study period were included ($n = 92$), resulting in 21,789,742 location data points. The data cleaning and processing steps resulted in total data removal of‘26%.

**Data Sheet 3 |** Validation of proximity identification protocol and additional results for different time durations and spatial thresholds, and temporal segmentation. We test and validate our algorithm for identifying and classifying proximity interactions against observed proximity events across a range of parameters (spatial threshold radii, $r = 1$–$5$ m; time duration, $t = 20$-$160$ s). We include additional results similar to the main paper for these additional parameter values, as well as alternative formats of temporal segmentation of the 28-day study period. In all cases, the results are qualitatively similar to the results given in the main paper and our conclusions hold.

## REFERENCES

1. Keeling L, Gonyou HW. *Social Behaviour in Farm Animals*. Wallingford, CT: CABI (2001).

2. Hasegawa N, Nishiwaki A, Sugawara K, Ito I. The effects of social exchange between two groups of lactating primiparous heifers on milk production, dominance order, behavior, and adrenocortical response. *Appl Anim Behav Sci.* (1997) 51:15–27. doi: 10.1016/S0168-1591(96)01082-9

3. Phillips CJC, Rind MI. The effects on production and behavior of mixing uniparous and multiparous cows. *J Dairy Sci.* (2001) 84:2424–9. doi: 10.3168/jds.S0022-0302(01)74692-9
4. Schirmann K, Chapinal N, Weary DM, Heuwieser W, von Keyserlingk MAG. Short-term effects of regrouping on behavior of prepartum dairy cows. *J Dairy Sci.* (2011) 94:2312–9. doi: 10.3168/jds.2010-3639
5. von Keyserlingk MAG, Olenick D, Weary DM. Acute behavioral effects of regrouping dairy cows. *J Dairy Sci.* (2008) 91:1011–6. doi: 10.3168/jds.2007-0532
6. Wey T, Blumstein DT, Shen W, Jordán F. Social network analysis of animal behaviour: a promising tool for the study of sociality. *Anim Behav.* (2008) 75:333–44. doi: 10.1016/j.anbehav.2007.06.020
7. de Freslon I, Martínez-López B, Belkhiria J, Strappini A, Monti G. Use of social network analysis to improve the understanding of social behaviour in dairy cattle and its impact on disease transmission. *Appl Anim Behav Sci.* (2019) 213:47–54. doi: 10.1016/j.applanim.2019.01.006
8. Croft DP, James R, Krause J. *Exploring Animal Social Networks*. New Jersey, NJ: Princeton University Press (2008).
9. Kim J, Hastak M. Social network analysis: characteristics of online social networks after a disaster. *Int J Inf Manag.* (2018) 38:86–96. doi: 10.1016/j.ijinfomgt.2017.08.003
10. Otte E, Rousseau R. Social network analysis: a powerful strategy, also for the information sciences. *J Inf Sci.* (2002) 28:441–53. doi: 10.1177/016555150202800601
11. CHOI J, Barnett G, CHON B-S. Comparing World City Networks: a network analysis of Internet backbone and air transport intercity linkages. *Glob Netw.* (2006) 6:81–99. doi: 10.1111/j.1471-0374.2006.00134.x
12. Makagon MM, McCowan B, Mench JA. How can social network analysis contribute to social behavior research in applied ethology? *Appl Anim Behav Sci.* (2012) 138:3. doi: 10.1016/j.applanim.2012.02.003
13. Croft D, Darden S, Wey T. Current directions in animal social networks. *Curr Opin Behav Sci.* (2016) 12:52–8. doi: 10.1016/j.cobeha.2016.09.001
14. Boyland NK, Mlynski DT, James R, Brent LJN, Croft DP. The social network structure of a dynamic group of dairy cows: from individual to group level patterns. *Appl Anim Behav Sci.* (2016) 174:1–10. doi: 10.1016/j.applanim.2015.11.016
15. Gygax L, Neisen G, Wechsler B. Socio-spatial relationships in dairy cows. *Ethology.* (2010) 116:10–23. doi: 10.1111/j.1439-0310.2009.01708.x
16. Hodges HR. *An Investigation of Social Structure in Housed Dairy Cows*. (2018) Available online at: http://repository.essex.ac.uk/23324/ (accessed July 11, 2020).
17. Neave HW, Costa JHC, Weary DM, von Keyserlingk MAG. Long-term consistency of personality traits of cattle. *R Soc Open Sci.* (2020) 7:191849. doi: 10.1098/rsos.191849
18. Boyland NK. *The Influence of Social Networks on Welfare and Productivity in Dairy Cattle*. (2015) Available online at: https://ore.exeter.ac.uk/repository/handle/10871/19360 (accessed July 11, 2020).
19. Rioja-Lang FC, Roberts DJ, Healy SD, Lawrence AB, Haskell MJ. Dairy cows trade-off feed quality with proximity to a dominant individual in Y-maze choice tests. *Appl Anim Behav Sci.* (2009) 117:159–64. doi: 10.1016/j.applanim.2008.12.003
20. Val-Laillet D, Rushen J, Keyserlingk M. The concept of social dominance and the social distribution of feeding-related displacements between cows. *Appl Anim Behav Sci.* (2007) 111:158–72. doi: 10.1016/j.applanim.2007.06.001
21. de Freslon I, Peralta JM, Strappini AC, Monti G. Understanding allogrooming through a dynamic social network approach: an example in a group of dairy cows. *Front Vet Sci.* (2020) 7:535. doi: 10.3389/fvets.2020.00535
22. Foris B, Zebunke M, Langbein J, Melzer N. Comprehensive analysis of affiliative and agonistic social networks in lactating dairy cattle groups. *Appl Anim Behav Sci.* (2019) 210:60–7. doi: 10.1016/j.applanim.2018.10.016
23. Schieltz JM, Okanga S, Allan BF, Rubenstein DI. GPS tracking cattle as a monitoring tool for conservation and management. *Afr J Range Forage Sci.* (2017) 34:173–7. doi: 10.2989/10220119.2017.1387175
24. Duncan S, Stewart TI, Oliver M, Mavoa S, MacRae D, Badland HM, et al. Portable global positioning system receivers: static validity and environmental conditions. *Am J Prev Med.* (2013) 44:e19–e29. doi: 10.1016/j.amepre.2012.10.013
25. Rice EM, Ferrell J, Vanzant E, Jackson J, Costa J. Real-time localization system for livestock dairy cattle: validation of static positioning in a commercial facility. In: *ASABE ASABE Annual International Virtual Meeting*. St. Joseph, MI (2020). doi: 10.13031/aim.202000797
26. Gygax L, Neisen G, Bollhalder H. Accuracy, and validation of a radar-based automatic local position measurement system for tracking dairy cows in free-stall barns. *Comput Electron Agric.* (2007) 56:23–33. doi: 10.1016/j.compag.2006.12.004
27. Tullo E, Fontana I, Gottardo D, Sloth KH, Guarino M. Technical note: validation of a commercial system for the continuous and automated monitoring of dairy cow activity. *J Dairy Sci.* (2016) 99:7489–94. doi: 10.3168/jds.2016-11014
28. Rocha LEC, Terenius O, Veissier I, Meunier B, Nielsen PP. Persistence of sociality in group dynamics of dairy cattle. *Appl Anim Behav Sci.* (2020) 223:104921. doi: 10.1016/j.applanim.2019.104921
29. Whitehead H. *Analyzing Animal Societies: Quantitative Methods for Vertebrate Social Analysis*. Chicago, IL: University of Chicago Press (2008).
30. Ballerini M, Cabibbo N, Candelier R, Cavagna A, Cisbani E, Giardina I, et al. Interaction ruling animal collective behavior depends on topological rather than metric distance: evidence from a field study. *Proc Natl Acad Sci.* (2008) 105:1232–7. doi: 10.1073/pnas.0711437105
31. Nagy M, Vásárhelyi G, Pettit B, Roberts-Mariani I, Vicsek T, Biro D. Context-dependent hierarchies in pigeons Máté Nagy. *Proc Natl Acad Sci USA.* (2013) 110:13049–54. doi: 10.1073/pnas.1305552110
32. Griffiths BE, Grove White D, Oikonomou G. A cross-sectional study into the prevalence of dairy cattle lameness and associated herd-level risk factors in england and wales. *Front Vet Sci.* (2018) 5:65. doi: 10.3389/fvets.2018.00065
33. Kofler J, Pesenhofer R, Landl G, Sommerfeld-Stur I, Peham C. Monitoring of dairy cow claw health status in 15 herds using the computerised documentation program Claw Manager and digital parameters. *Tierarztl Prax Ausg G Grosstiere Nutztiere.* (2013) 41:31–44. doi: 10.1055/s-0038-1623146
34. Thompson AJ, Weary DM, Bran JA, Daros RR, Hötzel MJ, von Keyserlingk MAG. Lameness and lying behavior in grazing dairy cows. *J Dairy Sci.* (2019) 102:6373–82. doi: 10.3168/jds.2018-15717
35. Tadich N, Flor E, Green L. Associations between hoof lesions and locomotion score in 1098 unsound dairy cows. *Vet J.* (2010) 184:60–5. doi: 10.1016/j.tvjl.2009.01.005
36. von Keyserlingk MAG, Barrientos A, Ito K, Galo E, Weary DM. Benchmarking cow comfort on North American freestall dairies: lameness, leg injuries, lying time, facility design, and management for high-producing Holstein dairy cows. *J Dairy Sci.* (2012) 95:7399–408. doi: 10.3168/jds.2012-5807
37. Amory JR, Barker ZE, Wright JL, Mason SA, Blowey RW, Green LE. Associations between sole ulcer, white line disease and digital dermatitis and the milk yield of 1824 dairy cows on 30 dairy cow farms in England and Wales from February 2003–November 2004. *Prev Vet Med.* (2008) 83:381–91. doi: 10.1016/j.prevetmed.2007.09.007
38. Archer SC, Green MJ, Huxley JN. Association between milk yield and serial locomotion score assessments in UK dairy cows. *J Dairy Sci.* (2010) 93:4045–53. doi: 10.3168/jds.2010-3062
39. Barker ZE, Amory JR, Wright JL, Mason SA, Blowey RW, Green LE. Risk factors for increased rates of sole ulcers, white line disease, and digital dermatitis in dairy cattle from twenty-seven farms in England and Wales. *J Dairy Sci.* (2009) 92:1971–8. doi: 10.3168/jds.2008-1590
40. Hernandez-Mendo O, von Keyserlingk MAG, Veira DM, Weary DM. Effects of pasture on lameness in dairy cows. *J Dairy Sci.* (2007) 90:1209–14. doi: 10.3168/jds.S0022-0302(07)71608-9
41. Thomas AP, Dipu MT. Lameness in dairy cattle: nutritional approaches for prevention and management. *Indian Vet J.* (2014) 12:18–22. Available online at: http://jivaonline.net/archive/download.php?file=pdf_222.pdf&id=222
42. Miguel-Pacheco GG, Thomas HJ, Huxley JN, Newsome RF, Kaler J. Effect of claw horn lesion type and severity at the time of treatment on outcome of lameness in dairy cows. *Vet J.* (2017) 225:16–22. doi: 10.1016/j.tvjl.2017.04.015
43. Leach KA, Tisdall DA, Bell NJ, Main DCJ, Green LE. The effects of early treatment for hindlimb lameness in dairy cows on four commercial UK farms. *Vet J.* (2012) 193:626–32. doi: 10.1016/j.tvjl.2012.06.043
44. Randall LV, Green MJ, Chagunda MGG, Mason C, Green LE, Huxley JN. Lameness in dairy heifers; impacts of hoof lesions present around first calving

on future lameness, milk yield and culling risk. *Prev Vet Med.* (2016) 133:52–63. doi: 10.1016/j.prevetmed.2016.09.006
45. Newsome RF, Green MJ, Bell NJ, Bollard NJ, Mason CS, Whay HR, et al. A prospective cohort study of digital cushion and corium thickness. Part 2: Does thinning of the digital cushion and corium lead to lameness and claw horn disruption lesions? *J Dairy Sci.* (2017) 100:4759–71. doi: 10.3168/jds.2016-12013
46. Tunstall J, Mueller K, Grove White D, Oultram JWH, Higgins HM. Lameness in beef cattle: UK farmers' perceptions, knowledge, barriers, and approaches to treatment and control. *Front Vet Sci.* (2019) 6:94. doi: 10.3389/fvets.2019.00094
47. Leach KA, Whay HR, Maggs CM, Barker ZE, Paul ES, Bell AK, et al. Working towards a reduction in cattle lameness: 1. Understanding barriers to lameness control on dairy farms. *Res Vet Sci.* (2010) 89:311–7. doi: 10.1016/j.rvsc.2010.02.014
48. Bran JA, Daros RR, von Keyserlingk MAG, Hötzel MJ. Lameness on Brazilian pasture based dairies—part 1: Farmers' awareness and actions. *Prev Vet Med.* (2018) 157:134–41. doi: 10.1016/j.prevetmed.2018.06.007
49. Dahl-Pedersen K, Foldager L, Herskin MS, Houe H, Thomsen PT. Lameness scoring and assessment of fitness for transport in dairy cows: agreement among and between farmers, veterinarians, and livestock drivers. *Res Vet Sci.* (2018) 119:162–6. doi: 10.1016/j.rvsc.2018.06.017
50. Weigele HC, Gygax L, Steiner A, Wechsler B, Burla J-B. Moderate lameness leads to marked behavioral changes in dairy cows. *J Dairy Sci.* (2018) 101:2370–82. doi: 10.3168/jds.2017-13120
51. Dairy. *AHDB*. Available online at: https://ahdb.org.uk/dairy#.Xv4SPihKg2w (accessed July 15, 2020).
52. Tadich N, Tejeda C, Bastias S, Rosenfeld C, Green LE. Nociceptive threshold, blood constituents and physiological values in 213 cows with locomotion scores ranging from normal to severely lame. *Vet J Lond Engl.* (2013) 197:401–5. doi: 10.1016/j.tvjl.2013.01.029
53. Barker ZE, Vázquez Diosdado JA, Codling EA, Bell NJ, Hodges HR, Croft DP, et al. Use of novel sensors combining local positioning and acceleration to measure feeding behavior differences associated with lameness in dairy cattle. *J Dairy Sci.* (2018) 101:6310–21. doi: 10.3168/jds.2016-12172
54. Palmer MA, Law R, O'Connell NE. Relationships between lameness and feeding behaviour in cubicle-housed Holstein–Friesian dairy cows. *Appl Anim Behav Sci.* (2012) 140:121–7. doi: 10.1016/j.applanim.2012.06.005
55. Blackie N, Bleach E, Amory J, Scaife J. Impact of lameness on gait characteristics and lying behaviour of zero grazed dairy cattle in early lactation. *Appl Anim Behav Sci.* (2011) 129:67–73. doi: 10.1016/j.applanim.2010.10.006
56. Alsaaod M, Römer C, Kleinmanns J, Hendriksen K, Rose-Meierhöfer S, Plümer L, et al. Electronic detection of lameness in dairy cows through measuring pedometric activity and lying behavior. *Appl Anim Behav Sci.* (2012) 142:134–41. doi: 10.1016/j.applanim.2012.10.001
57. Vázquez Diosdado JA, Barker ZE, Hodges HR, Amory JR, Croft DP, Bell NJ, et al. Space-use patterns highlight behavioural differences linked to lameness, parity, and days in milk in barn-housed dairy cows. *PLoS ONE.* (2018) 13:e208424. doi: 10.1371/journal.pone.0208424
58. Cramer MC, Stanton AL. Associations between health status and the probability of approaching a novel object or stationary human in preweaned group-housed dairy calves. *J Dairy Sci.* (2015) 98:7298–308. doi: 10.3168/jds.2015-9534
59. Cramer MC, Ollivett TL, Stanton AL. Associations of behavior-based measurements and clinical disease in preweaned, group-housed dairy calves. *J Dairy Sci.* (2016) 99:7434–43. doi: 10.3168/jds.2015-10207
60. Fogsgaard KK, Røntved CM, Sørensen P, Herskin MS. Sickness behavior in dairy cows during *Escherichia coli* mastitis. *J Dairy Sci.* (2012) 95:630–8. doi: 10.3168/jds.2011-4350
61. Dittrich I, Gertz M, Krieter J. Alterations in sick dairy cows' daily behavioural patterns. *Heliyon.* (2019) 5:e02902. doi: 10.1016/j.heliyon.2019.e02902
62. Belaid MA, Rodríguez-Prado M, Rodríguez-Prado DV, Chevaux E, Calsamiglia S. Using behavior as an early predictor of sickness in veal calves. *J Dairy Sci.* (2020) 103:1874–83. doi: 10.3168/jds.2019-16887
63. Goldhawk C, Chapinal N, Veira DM, Weary DM, von Keyserlingk MAG. Prepartum feeding behavior is an early indicator of subclinical ketosis. *J Dairy Sci.* (2009) 92:4971–7. doi: 10.3168/jds.2009-2242
64. Patbandha TK, Mohanty TK, Layek SS, Kumaresan A, Behera K. Application of pre-partum feeding and social behaviour in predicting risk of developing metritis in crossbred cows. *Appl Anim Behav Sci.* (2012) 139:10–7. doi: 10.1016/j.applanim.2012.03.014
65. Sepúlveda-Varas P, Proudfoot KL, Weary DM, von Keyserlingk MAG. Changes in behaviour of dairy cows with clinical mastitis. *Appl Anim Behav Sci.* (2016) 175:8–13. doi: 10.1016/j.applanim.2014.09.022
66. Galindo F, Broom DM. The effects of lameness on social and individual behavior of dairy cows. *J Appl Anim Welf Sci.* (2002) 5:193–201. doi: 10.1207/S15327604JAWS0503_03
67. Overton MW, Sischo WM, Temple GD, Moore DA. Using time-lapse video photography to assess dairy cattle lying behavior in a free-stall barn. *J Dairy Sci.* (2002) 85:2407–13. doi: 10.3168/jds.S0022-0302(02)74323-3
68. *R: The R Project for Statistical Computing*. Available online at: https://www.r-project.org/ (accessed July 11, 2020).
69. RStudio Team. *RStudio: Integrated Development for R*. RStudio (2020). Available online at: https://rstudio.com/products/rstudio/ (accessed July 11, 2020).
70. Csardi G, Nepusz T. *igraph: Network Analysis and Visualization*. (2020). Available online at: https://CRAN.R-project.org/package=igraph (accessed July 11, 2020).
71. Farine D. A guide to null models for animal social network analysis. *Methods Ecol Evol.* (2017) 8:1309–20. doi: 10.1111/2041-210X.12772
72. Farine DR, Whitehead H. Constructing, conducting and interpreting animal social network analysis. *J Anim Ecol.* (2015) 84:12418. doi: 10.1111/1365-2656.12418
73. Phipson B, Smyth GK. Permutation p-values should never be zero: calculating exact p-values when permutations are randomly drawn. *Stat Appl Genet Mol Biol.* (2010) 9:1585. doi: 10.2202/1544-6115.1585
74. Mantel N. The detection of disease clustering and a generalized regression approach. *Cancer Res.* (1967) 27:209–20.
75. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. *vegan: Community Ecology Package*. (2019). Available online at: https://CRAN.R-project.org/package=vegan (accessed October 30, 2020).
76. Krause J, Croft DP, James R. Social network theory in the behavioural sciences: potential applications. *Behav Ecol Sociobiol.* (2007) 62:15–27. doi: 10.1007/s00265-007-0445-8
77. Salau J, Lamp O, Krieter J. Dairy cows' contact networks derived from videos of eight cameras. *Biosyst Eng.* (2019) 188:106–13. doi: 10.1016/j.biosystemseng.2019.10.018
78. Allen JD, Hall LW, Collier RJ, Smith JF. Effect of core body temperature, time of day, and climate conditions on behavioral patterns of lactating dairy cows experiencing mild to moderate heat stress. *J Dairy Sci.* (2015) 98:118–27. doi: 10.3168/jds.2013-7704
79. Herbut P, Angrecka S. Full article: relationship between THI level and dairy cows' behaviour during summer period. *Ital J Anim Sci.* (2018) 17:226–33. doi: 10.1080/1828051X.2017.1333892
80. Shahriar MdS, Smith D, Rahman A, Henry D, Bishop-Hurley G, Rawnsley R, et al. Heat event detection in dairy cows with collar sensors: an unsupervised machine learning approach. In: *2015 IEEE SENSORS*. Busan (2015). p. 1–4. doi: 10.1109/ICSENS.2015.7370528
81. Shahriar MdS, Smith D, Rahman A, Freeman M, Hills J, Rawnsley R, et al. Detecting heat events in dairy cows using accelerometers and unsupervised learning. *Comput Electron Agric.* (2016) 128:20–6. doi: 10.1016/j.compag.2016.08.009
82. James R, Croft DP, Krause J. Potential banana skins in animal social network analysis. *Behav Ecol Sociobiol.* (2009) 63:989–97. doi: 10.1007/s00265-009-0742-5
83. Dantzer R. Cytokine-induced sickness behavior: where do we stand? *Brain Behav Immun.* (2001) 15:7–24. doi: 10.1006/brbi.2000.0613
84. Galindo F, Broom DM. The relationships between social behaviour of dairy cows and the occurrence of lameness in three herds. *Res Vet Sci.* (2000) 69:75–9. doi: 10.1053/rvsc.2000.0391

85. Groenevelt M, Main DCJ, Tisdall D, Knowles TG, Bell NJ. Measuring the response to therapeutic foot trimming in dairy cows with fortnightly lameness scoring. *Vet J.* (2014) 201:283–8. doi: 10.1016/j.tvjl.2014.05.017
86. Boyland NK, James R, Mlynski DT, Madden JR, Croft DP. Spatial proximity loggers for recording animal social networks: consequences of inter-logger variation in performance. *Behav Ecol Sociobiol.* (2013) 67:1877–90. doi: 10.1007/s00265-013-1622-6
87. Harris NR, Johnson DE, McDougald NK, George MR. Social associations and dominance of individuals in small herds of cattle. *Rangel Ecol Manag.* (2007) 60:339–49. doi: 10.2111/1551-5028 (2007) 60[339:SAADOI]2.0.CO;2
88. Gutmann AK, Špinka M, Winckler C. Long-term familiarity creates preferred social partners in dairy cows. *Appl Anim Behav Sci.* (2015) 169:1–8. doi: 10.1016/j.applanim.2015.05.007
89. Gutmann AK, Špinka M, Winckler C. Do familiar group mates facilitate integration into the milking group after calving in dairy cows? *Appl Anim Behav Sci.* (2020) 229:105033. doi: 10.1016/j.applanim.2020.105033
90. *Managing Cow Lactation Cycles*. Available online at: http://www.thecattlesite.com/articles/4248/managing-cow-lactation-cycles/ (accessed July 14, 2020).
91. Croft DP, Madden JR, Franks DW, James R. Hypothesis testing in animal social networks. *Trends Ecol Evol.* (2011) 26:502–7. doi: 10.1016/j.tree.2011.05.012

**Conflict of Interest:** NB is employed by company Bos International Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 15 December 2020
doi: 10.3389/fvets.2020.589274

# Psychological and Physiological Stress in Hens With Bone Damage

*Neža Rokavec and Manja Zupan Šemrov**

*Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Domžale, Slovenia*

Abnormalities in bone development in humans and non-humans can lead to impaired physical and psychological health; however, evidence is lacking regarding the role of individual psychosocial factors in the development of poor bone conditions. Addressing this lack of knowledge, we used low-productive laying hens ($n = 93$) and assessed behavioral responses to an open-field test [at 17, 18, 29, 33 weeks of age (wa)], an aerial predator test (at 39 wa), and a social reinstatement test (at 42 wa). Bone condition was assessed using a palpation technique on five occasions (at 16, 29, 33, 45, 58 wa), with half of the hens experiencing damage (deviations, fractures, or both) at 29 wa and all hens by 58 wa. Corticosterone (CORT) concentration in feathers (at 16, 33, 58 wa) and body weight (at 23, 47, 58 wa) were also investigated. We hypothesized that lighter birds (at 23 wa) with higher CORT (at 16 wa) and open field-induced fear collected before the onset of lay (at 17 and 18 wa) are associated with a worse bone condition when in lay. We also hypothesized that those birds with more damage at the peak of laying (at 29 wa) would be lighter at 47 and 58 wa and more fearful by showing higher open field-induced (at 29 and 33 wa) and predator-induced fear responses, however, acting less socially toward conspecifics. These hens were also expected to have higher CORT (at 33 and 58 wa). Our results show no association between open-field fear level and fear behavior, CORT concentration, or body weight on the one hand (all measured before starting to lay) and bone damage at 29 wa on the other. When in lay, bone damage was associated with more pecking and less crossing zones when faced with an open-field situation at 29 wa and improved sociality at 42 wa. This study provides the first evidence of a relationship of bone health with fear, sociality, and stress response. When in poor bone condition, our hens had enhanced psychological stress measured by fear behavior reactivity but not physiological stress measured as feather CORT concentration.

**Keywords: keel bone, poultry, stress physiology, behavior, body development, affective state**

OPEN ACCESS

***Edited by:***
*Michael Toscano,*
*University of Bern, Switzerland*

***Reviewed by:***
*Sabine G. Gebhardt-Henrich,*
*University of Bern, Switzerland*
*Christina Barbara Rufener,*
*University of California, Davis, United States*
*Rebecca E. Nordquist,*
*Utrecht University, Netherlands*

***Correspondence:**
*Manja Zupan Šemrov*
*manja.zupansemrov@bf.uni-lj.si*

***Specialty section:***
*This article was submitted to Animal Behavior and Welfare, a section of the journal Frontiers in Veterinary Science*

***Received:*** *30 July 2020*
***Accepted:*** *12 November 2020*
***Published:*** *15 December 2020*

***Citation:***
*Rokavec N and Zupan Šemrov M (2020) Psychological and Physiological Stress in Hens With Bone Damage. Front. Vet. Sci. 7:589274. doi: 10.3389/fvets.2020.589274*

## INTRODUCTION

Bone disease, such as osteoporosis in humans, is often seen as a silent disorder until it causes fractures (1). Yet, the consequence of such disease can have a major impact on individuals such as a decrease in physical and psychological health. Many humans who suffer from bone fractures experience significant pain and weight loss; they may lose the ability to stand and walk (2) or may be immobilized by a fear of falling (3) or even begin to feel isolated and helpless (2). On top of these effects, an increase in indirect costs [e.g., lost productivity for patients and caregivers (2) and increased stress level (4)] has recognized.

Animal welfare scientists agree that laying hens suffer from a variety of welfare problems, including the keel bone damage (KBD) (5), which is estimated to reach a prevalence of between 30 and 90% by 45 weeks of age (wa) when the ossification of the keel bone (KB) is completed (6–8). KBD includes both fractures and deviations (9). Unlike fractures, which usually happen during an isolated event such as crashes/collisions during flight or uncontrolled landings and takeoffs (9), the development of deviations happens over a period of time as an outcome of bone remodeling in response to regular loading pressure during roosting (7). Bone damage is known to affect a broad spectrum of issues in the poultry industry, including egg production (8, 10, 11), water intake (12), body weight (6), deformation of breast muscle (12), and to cause welfare problems (9) including pain (13). In 1868, Darwin became the first to document (14) that egg-producing domestic fowl, laying fewer eggs than the hens nowadays, exhibited KBs that were moderately crooked or extremely deformed. In the early 1990s, crooked keels in laying hens were ascribed to hereditary disease (15), rickets (16), faulty metabolism, or a slow process of ossification (17). Despite this, only recently has research intensively focused on looking at the underlying causes and consequences of KBD in commercial laying hens.

Recent findings (10, 12, 18) reveal that it is likely that production is just one of many factors affecting bone integrity, explaining why some studies found no effect on egg production (6, 12) or body weight (19). By reviewing several research studies, Riber et al. (9) summarized that psychological stress factors may be related to bone damage and that KBD promotes the expression of negative states. In layers, investigating negative affective states focused mainly on pain (13, 19) or the fear level (20–22), often related to fear of humans (22), while for positive states, it concentrated on assessing social behavior (21). Studies also found that bone damage affects not only welfare but physiological parameters (10), although the problem's multifactorial nature makes it difficult to study the underlying causes and consequences of KBD in commercial laying hens.

To date, insufficient longitudinal data have been available to link bone damage and emotional consequences or to investigate the hypothesis that the affective state may impact damage. Riber and Hinrichsen (23) suggested, albeit have not yet clearly demonstrated, that a link exists between injurious pecking damage, bone damage, and fearfulness. Recently, by investigating changes in the hippocampus in a small number of commercial Lohmann Brown hens (15 hens with severe and nine hens with minimal KB fractures) in an aviary system, Armstrong et al. (24) found that hens with KB fractures are more likely to experience negative affective states that last for at least 3–4 weeks. In line with this and the fact that fearfulness of an individual, which is a known measure of psychological stress and thus, a negative affective state, could affect physical health (25) and its sensitivity to physiological stress (26), our main objective was to investigate the relationships between the affective state recorded during behavioral testing and the development of bone condition, corticosterone (CORT) concentration, and body weight. Levels of CORT deposited in feathers were analyzed to provide a measure of longer term physiological stress [i.e., (26, 27)]. Slovenian locally adapted laying hens of the Styrian breed (n = 93) were subjected to standardized test situations [i.e., open-field test (OFT), aerial predator test (APT), social reinstatement test (SRT)], and the level of fear and sociality were measured. We chose this particular strain of bird to improve our understanding of the behavioral and stress responses of hens with low egg production and good resistance to diseases (28); their bone condition is expected to be less likely poor and to show greater variation in behavioral responses compared to highly productive hens that have been intensively selected. Moreover, thus far, there are no data on the association between the prevalence of KBD in non-commercial chicken breeds and affective states. Assuming lighter birds are more prone to show fear behavior and be fearful (29), fearful birds have a higher risk of injuries (30), and bone development depends on the concentration of glucocorticoids in humans (2) and animals (18, 31), we first hypothesized that lighter individuals that show more fear-related characteristics and a higher stress-induced CORT before starting to lay will have a poorer bone condition at a later time point and, second, that those birds with more damage at the peak of laying will show more fear but act less socially toward conspecifics and have a lower body weight and a higher CORT. The latest hypothesis was derived from human studies (2–4) but also from the suggestion that when small prey animals are subjected to fear stimuli such as predator-like stimulus, this may elevate long-term stress and defensive responses and may lead to future stress-induced weight loss (32).

# MATERIALS AND METHODS

## Animals and Housing

The experiment was conducted from October 2017 to August 2018 at the Krumperk Educational and Research Centre, University of Ljubljana, Biotechnical Faculty. Randomly selected pullets ($n = 93$) and cockerels ($n = 15$) were obtained from a commercial flock of a basic floor-rearing system at 16 wa and transported to the laying pen (l × w = 865 × 496 cm). From 16 to 58 wa, the flock was kept in this barn system with wood shavings (7-cm depth) and started to lay at 23 wa. To allow recognition, all females were marked with leg rings. The laying pen was divided by a wire mesh into a smaller (l × w = 865 × 186 cm) and a larger (l × w = 865 × 310 cm) area linked by an always-open door. Light was provided by two bulbs according to a 14:10 h light:dark cycle. Chickens had free access to a standard commercial layer diet from three round feeders (at 27 cm height) and water from drinking lines (at 37 cm height) with 25 water nipples in the smaller area and 29 in the larger area. Three wooden perches were placed in the middle of the larger area, each with dimensions of 190 × 4 × 6 cm, placed at a height of 66 cm above the ground. The pen contained two metal nest box lines at a height of 50 cm above the ground when measured from the lower line, with 14 nest boxes each (w × d × h = 30 × 30 × 30 cm) and three wooden perches (l × w × h = 200 × 4 × 2 cm) in front. The available perch space was 12.9 cm per bird. Two automatic axial propeller fans were used to draw air out of the building through the wall vents (negative pressure

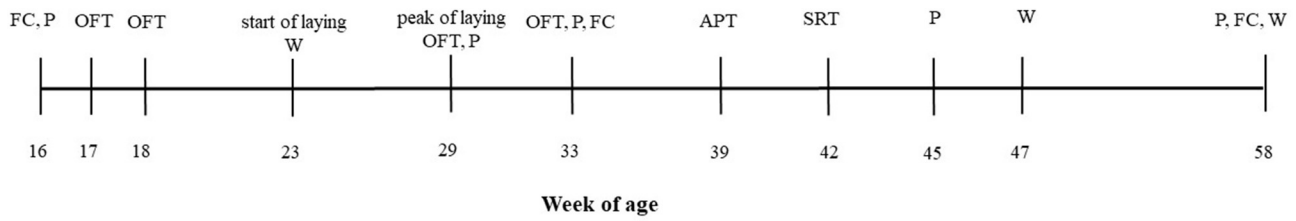


**FIGURE 1 |** The timeline of the measurements taken. FC, feather collection; P, palpation; W, body weight; OFT, open-field test; APT, aerial predator test; SRT, social reinstatement test.

ventilation), and two air inlets were used to ensure fresh air entered the barn.

## Experimental Design

**Figure 1** illustrates the different experimental procedures carried out over a period of 42 weeks, during which the hens were individually weighed (at 23, 47, 58 wa), exposed to three different behavioral tests, and palpated to record KB status. To obtain a retrospective measure of the long-term stress experienced by the birds during feather growth (27, 33), feathers were taken at three time points for analysis of CORT concentrations. These procedures are explained in more detail in the following sections and were chosen to investigate the relationships between fear as an indicator of psychological stress, CORT as an indicator of physiological stress, and KBD as well as body weight as a physical condition.

## Palpation of the Keel Bone

The presence of both fractures and deviations of the KB was assessed by palpation on five occasions (at 16, 29, 33, 45, and 58 wa) using the Simplified Keel Assessment Protocol (SKAP) palpation system (34). At 16 and 29 wa, only the presence or absence of damage was recorded, whereas at 33, 45, and 58 wa, the type of damage (deviation, fracture, or both) was also specified. The person assessing the damage was trained on how to palpate hens during a 2-day course at the University of Bern in 2017. The study of this training school revealed that training with radiographs improved palpation accuracy (35). On each assessment occasion, hens were taken from the pen in random order and transferred to a nearby room. Each hen was held in the observer's arms in the position of a cradle and the ventral and lateral surfaces of the KB were palpated by running the forefinger and thumb up and down the bone.

## Feather Collection and Corticosterone Analyses

Feather collection was done by cutting the primary third feather of the wing from each hen. The first feather was representative of the period before the onset of lay and cut out at 16 wa. The second feather was cut at 33 wa, i.e., 4 weeks after the peak of lay, and the final one at the end of the experiment (58 wa). The first and the third feathers (that grew out between 16 and 58 wa) were taken from the left wing and the second feather from the right wing. Feathers were stored separately in a paper envelope and kept on a shelf at ambient indoor temperature before analysis. A methanol-based extraction technique was used to extract CORT from feathers [adjusted after Bortolotti et al. (27)]. The feathers were prepared by cutting vanes into pieces with scissors. From each feather, 30 mg was used and put into a test tube. Then, 10 ml of methanol gradient grade for liquid chromatography (Merck KGaA, Darmstadt, Germany) was added, and the samples were placed in a sonicating water bath at room temperature for 30 min, followed by incubation at 50°C overnight in a shaking water bath. The methanol was then separated from the feather material by basic gravity filtration using a cellulose filter paper in the filtration funnel. The feather remnants, original sample vial, and filtration material were washed twice with ∼2.5 ml of additional methanol; the washes were added to the original methanol extract. The methanol extract was placed in a 50°C water bath and subsequently evaporated in a fume hood under nitrogen gas. Evaporation of the samples was completed within a few hours, and the extract residues were reconstituted in 500 µl of 15% methanol. Reconstituted samples were frozen at−20°C until analyzed for CORT. The concentration of the samples (nmol/L extract) was assayed using the commercially available ELISA kit (DE4164, Kiel, Germany). The test procedure followed standard methods. While calculating the CORT concentration in feathers (pM/g), the dilution factor (16.67) was taken into account. The CORT concentration represented three measures (CORT at 16 wa represented the storage between 0 and 16 wa; CORT at 33 wa represented the storage between 0 and 33 wa; CORT at 58 wa was the sum of CORT at 16 wa and CORT at 33 wa).

## Behavioral Tests

Starting at 17 wa, the hens were subjected to several behavioral tests. The tests were performed in the same order for each hen, i.e., first the OFTs, then APT, and finally the SRT. The OFT was performed four times at different ages in order to investigate intra-situation coping responses. As for inter-situation behavioral responses that may represent generalized fearfulness, APT and SRT were performed at later ages but were not tested in weeks in which palpation or feather collection were carried out in order to avoid confounding the behavioral readouts. Consistency of behaviors in different tests was investigated. Hens were individually caught from the pen, each time from a different location in order to avoid biases in the test order as less fearful or slower birds are often picked

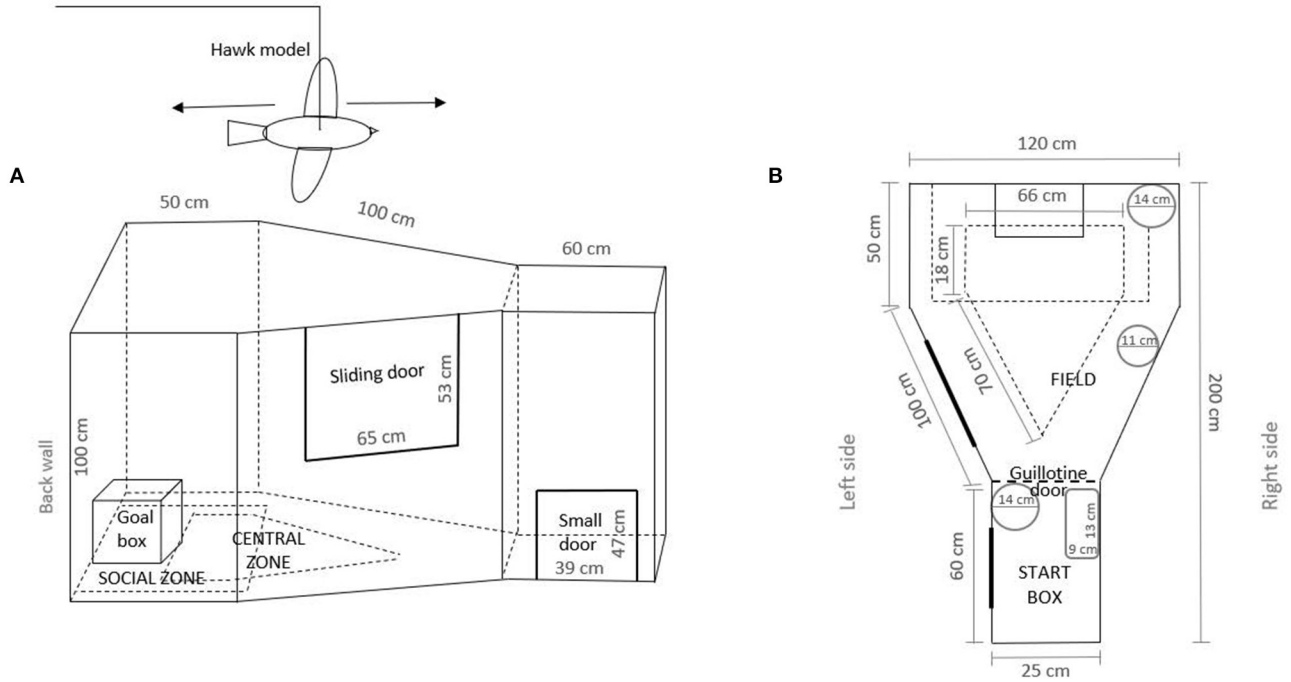


**FIGURE 2 |** Experimental apparatus showing **(A)** side view and **(B)** top view.

first, and brought to the test apparatus in one person's hands. Their behaviors were recorded using direct observations by one observer. Recorded by stopwatch, timing started 30 s after being placed in the test apparatus.

## Test Apparatus

The apparatus (**Figure 2**) was a weather-resistant black plywood (T-fix) trapezoid-like arena isolated from humans and animals [adjusted after de Haas et al. (36)]. It was located in a 474 (l) × 360 (w) × 258 cm (h) room next to the laying pen and illuminated with a light bulb of 206.9 lx (measured at the hen's head). An extra light was placed 140 cm above the apparatus to help track the test hen and ensure visibility. A hen was placed in the start box through a small door and then introduced into the field arena when the guillotine door of the start box was opened. Observations started when the guillotine door was opened. To measure movement, the arena was divided into zones (i.e., central and social zone) marked with black tape. A human could gain access through the sliding door to catch a bird and to clean (i.e., vacuuming feathers and wood shavings and absorbing feces with cellulose) the area after each testing. By using mirrors, the observer standing behind the start box was out of view of the test subjects so as not to influence their behavior. The circular and rectangular mirrors were placed above the guillotine door of the start box. Other circular mirrors were on the back wall and on the right side of the apparatus. Part of the scoring criteria included flying out of the arena, which is why the top of the arena was not covered.

## Open-Field Test

The hens ($n$ = 93) were individually exposed to an OFT at 17, 18, 29, and 33 wa between 9:00 and 15:00 h. Each of them lasted 3 min. At 10, 20, 30, 40, 50, and 60 s after opening the guillotine door, the birds' fear responses were categorized as calm, ambiguous, fearful, and highly fearful according to specific behaviors detailed in predetermined selection criteria (**Table 1**), and the fear scores were averaged across the six observations.

Other fear behaviors (**Table 2**) were recorded for 3 min of testing using continuous sampling. Pecking and preening were added because they frequently occurred during the pilot study.

**TABLE 1 |** Selection criteria for fear responses in the open field test.

| Fear response | Behavior | Fear score |
|---|---|---|
| Calm animal | Exploring, standing or walking, short or normal length of the neck, and no vocalizing or vocalizing quietly (calm, low) | 20 |
| Ambiguous animal | Standing or walking, neck stretched, head flicks, and no vocalizing or vocalizing quietly (calm, low) | 40 |
| Fearful animal | Standing or walking, neck stretched, head flicks, and vocalizing loudly | 60 |
| Highly fearful animal | Escape, attempting to escape, and vocalizing loudly or no vocalizing. The bird is completely still (freeze behavior). | 80 |

*Fear scores were adapted from Agnvall et al. (37). Fear responses and behaviors were newly defined.*

### Aerial Predator Test

Behavior was observed during a simulated aerial predator attack that was carried out once at 39 wa, between 9:00 and 14:00 h, to investigate the initial response to a potential natural predator. For behaviors (**Table 2**), we used instantaneous sampling with 10-s intervals during 2 min of testing, while continuous sampling was used for latency to escape. In order to obtain a baseline of the behavior, the animals were first observed undisturbed. After 1 min of opening the guillotine door, a hawk-silhouette model (**Figure 2**) (measuring 41.0 × 22.4 cm) made out of brown-colored plywood (natural color of the falcon) was pulled back and forth along a string starting 140 cm above the testing room floor, 15 cm from the back wall of the apparatus. The model passed through the arena's 340 cm in 3 s. Before and after the simulated overflight, the hawk silhouette was hidden behind a gray curtain.

### Social Reinstatement Test

At 42 wa, the hens' level of sociality (motivation to be with conspecifics) was measured between 8:00 and 12:00 h. Beside the back wall of the arena, one stimulus hen familiar to the test bird (one of 93 test hens) and of the same age, was kept in a wooden framed box (**Figure 2**) of 30 (l) × 40 (d) × 40 (h) made of wire mesh. The stimulus bird was changed after each test. An area close to the social companion was defined as a social zone, marked with black tape at 25 cm around the goal box. This distance was chosen according to Dawkins (38), who claims that social recognition in hens may only occur at distances <30 cm. The hens were tested once. The test procedure was as follows: when the test started, the behaviors described in **Table 2** were observed for 3 min using continuous sampling.

## Statistical Analysis

Five hens unexpectedly and unrelated to the experiment died before 58 wa and were excluded from certain analyses. Further, feather CORT concentration was missing for four hens at 16 wa and for one hen at 33 wa. The statistical analysis was performed using the SAS/STAT software, version 9.4, of the SAS System for Windows © 2002–2012 SAS Institute Inc. The normal distribution for quantitative traits was determined by the Shapiro–Wilk test. All reported $P \leq 0.05$ were classified as statistically significant while <0.06 as with a strong tendency.

Considering the behavioral observations, latencies to leave the start box and to reach the central zone among repetitions of OFT were analyzed in a survival analysis context (39) by the PHREG procedure, where non-events were treated as right-censored data. Preening and pecking as well as APT behaviors were calculated first as frequencies per hen and then as number of times per time period for all the tested birds. A difference between the behaviors observed in OFTs, as well as before and after a hawk appearance in APT, was investigated using the chi-square test. Consistency was computed for behaviors in repeated OFTs as suggested by

**TABLE 2 |** Ethogram of the behaviors recorded during the tests and their descriptions.

| Test | Behavior | Description |
|---|---|---|
| **OFT** | | |
| | Latency to leave the start box$^{D}$ | Length of time from the start of testing to stepping in the field with both feet |
| | Latency to reach the central zone $^{D}$ | Length of time when both feet reach into the central zone |
| | Crossing the central zone$^{F}$ | Defines how often the hen crosses the central zone |
| | Preening$^{F}$ | Defines how often the hen moves its head in a smoothing motion over the body |
| | Pecking$^{F}$ | Defines how often the hen pecks on the ground or at the wall of the platform as visual inspection |
| **APT** | | |
| | Activity$^{F}$ | Defines how often the hen has a relaxed body stance, short or normal length of the neck (when she moves, stands, or sits) and does not vocalize or vocalizes quietly |
| | Freeze$^{F}$ | Defines how often the hen is completely still* |
| | Escape attempt$^{F}$ | Defines how often the hen tries to escape, i.e., constantly looks up at the top of the platform with neck stretched or tries to fly out |
| | Being alert$^{F}$ | Defines how often the hen has an alert body stance with neck stretched (when she moves, stands, or sits) and does not vocalize or vocalizes loudly |
| | Latency to escape$^{D}$ | Length of time from the start of testing to the platform breakout |
| **SRT** | | |
| | Vocalization$^{F}$ | Defines how often the hen vocalizes |
| | Latency to vocalize$^{D}$ | Length of time to the first sound the hen makes |
| | Latency to escape$^{D}$ | Length of time from the start of testing to the platform breakout |
| | Latency to leave the start box$^{D}$ | Length of time from the start of testing to stepping in the field with both feet |
| | Latency to reach the social zone$^{D}$ | Length of time when at least one of the feet reaches the social zone or a hen jumps from the field on the cage located in the social zone |
| | Duration in social zone$^{D}$ | Time spent in the social zone* |

*$^{F}$, behavior recorded as frequency; $^{D}$, behavior recorded as duration; OFT, open-field test; APT, aerial predator test; SRT, social reinstatement test. *descriptions were adjusted after Agnvall et al. (37).*

Nakagawa and Schielzeth (40), where behaviors were treated as binary variables with 1 (i.e., event) and 0 (i.e., non-event). A multinomial overdispersion model was used in the GLIMMIX procedure, and consistencies are presented on a latent scale.

In the following step, the KBD as a binary outcome (1, damage; 0, no damage) and CORT concentration as a continuous variable at different ages were analyzed by the FREQ procedure using the CMH option in the TABLES statement or by the MIXED procedure with a hen treated as a random effect, respectively. Since the majority of hens experienced KBD at 33 wa (80.6%) and all hens at 58 wa (100.0%), it was only possible to use KBD data at the peak of lay (29 wa), where 50% of hens were found with KBD for further analysis. Using the LOGISTIC procedure with modeling probability that KBD is 1, we investigated if the average fear score at 17 (OFT1) and 18 wa (OFT2) and fear-related behavior responses in OFT1 and OFT2, body weight at 23 wa, and CORT at 16 wa affected KBD. We were further interested to know if the presence/absence of KBD was related to the average fear score at 29 (OFT3) and 33 wa (OFT4), body weight at 47 and 58 wa, CORT at 33 and 58 wa, and the behavior responses displayed in OFT, APT, and SRT. In the original models, all of the tested variables were included, but those found not significant for KBD were removed. Wald chi square statistics was provided for results deriving from the LOGISTIC procedure.

# RESULTS

## Behavioral Tests

### Open Field Test

With repetitive exposure to the OFT, more hens left the start box ($\chi^2 = 25.43$, df = 4, $P < 0.0001$) and did so faster ($\chi^2 = 72.02$, $P < 0.0001$; data not shown). The highest number of hens reached the central zone in OFT3 ($\chi^2 = 17.75$, df = 3, $P = 0.0005$), and they did so more frequently in OFT3 and OFT4 than in OFT1 and OFT2 ($\chi^2 = 40.62$, df = 3, $P < 0.0001$; data not shown). There were six hens that did not leave the start box in any of the four repetitions. The frequencies of preening (from OFT1 to OFT4 test; 1: $n = 57$, 2: $n = 104$, 3: $n = 72$, 4: $n = 62$; $\chi^2 = 18.13$, df = 3, $P = 0.0004$) and pecking (from OFT1 to OFT4 test; 1: $n = 104$, 2: $n = 150$, 3: $n = 127$, 4: $n = 145$; $\chi^2 = 9.89$, df = 3, $P = 0.02$) also differed among repetitions.

Consistency of behaviors treated as binary traits in repeated OFTs was low to moderate. Leaving the start box over four repetitions of OFT had consistency of 0.181, and with inclusion of SRT, it increased to 0.188. If only records from the rearing period were considered, consistency was 0.211, and for records from only the laying period, it was 0.561. Consistency for reaching the central zone was lower than consistency for leaving the start box, with higher values in the rearing and laying period separately compared to the inclusion of all four repetitions of OFT. Preening had the highest consistency of the behaviors observed; 0.345 for four repetitions of OFT, 0.442 in the rearing and 0.493 in the laying period. Consistency for pecking was 0.159 for four repetitions of OFT, 0.093 in the rearing and 0.289 in the laying period.

### Aerial Predator Test

The hens' behavior differed when comparing responses before and after the appearance of the hawk. More exploring before its appearance (283 vs. 172 times; $\chi^2 = 27.08$, df = 1, $P < 0.0001$) and less standing alert (139 vs. 202 times; $\chi^2 = 11.64$, df = 1, $P = 0.0006$) were observed. No differences were observed in freezing behavior (89 times before vs. 111 times after; $\chi^2 = 2.42$, df = 1, $P = 0.12$) and escape attempts (31 times before vs. 32 times after; $\chi^2 = 0.02$, df = 1, $P = 0.90$). Some hens froze (before: $n = 22$; after: $n = 27$), and others tried to escape the test apparatus ($n = 18$), but few managed to escape (before: $n = 5$; after: $n = 3$).

### Social Reinstatement Test

In the SRT, hens ($n = 78$) left the start box in 23.85 ± 3.30 s, 53 hens reached the social zone in 63.21 ± 6.12 s and stayed in the zone for 100.75 ± 6.98 s. They needed 55.42 ± 7.07 s to start vocalizing ($n = 48$ hens), with the maximum number of events per hen being 18. Some hens ($n = 10$) escaped from the test apparatus with a latency of 49 ± 14.79 s.

## Keel Bone Damage

The number of hens exhibiting bone damage (deviations and fractures combined) increased with age (Mantel–Haenszel $\chi^2 = 21.86$, df = 1, $P < 0.0001$), with most hens without KBD at 16 wa (6.5%), half of the hens with KBD at 29 wa (50.4%), almost all hens at 45 wa (94.6%), and all hens showing KBD at 58 wa (100%). CORT concentration was also found to increase with age (mean ± SD; 16 wa = 44.78 ± 16.07; 33 wa = 67.83 ± 29.73; 58 wa = 96.95 ± 25.03; $F = 696.81$, df = 2, $P < 0.0001$). Hens weighed 1435.05 ± 164.11 g at 23 wa, 1795.22 ± 213.24 g at 47 wa, and 1825.39 ± 226.33 g at 58 wa.

Before starting to lay and by using a logistic regression model, we found that body weight affected KBD by lighter hens showing more bone damage (Wald $\chi^2 = 4.65$, $P = 0.03$), but biological significance was negligible (for a 1 kg heavier hen probability increased by only 0.3%). CORT stored from 0 to 16 wa had no relationship to KBD at 29 wa (Wald $\chi^2 = 0.17$, $P = 0.68$) nor had the average fear score at 17 (Wald $\chi^2 = 1.28$, $P = 0.26$) or 18 wa (Wald $\chi^2 = 0.49$, $P = 0.48$). When in lay, pecking and frequency of crossing zones at 29 wa in OFT3 as well as latency to leave starting arena in SRT showed a relationship with KBD (**Table 3**). Hens with bone damage at 29 wa were pecking more but crossing zones less in the OFT and reaching the testing arena faster in SRT. No other relationships were found including those between KBD and average fear scores (data not shown).

# DISCUSSION

This study used fowl as an animal model to investigate the relationship between fear and stress responses and bone health. We found multiple relationships. Although we cannot confirm that the patterns we observed in the predator- and open field-induced fear situations are individual behavioral strategies stable over a longer time, our results support the existence of a relationship between psychological stress experienced as fear and the development of physical health reflected in bone condition.

**TABLE 3 |** Behavior responses as frequency (mean ± SE) associated with KBD at 29 week of age (wa) in OFT and SRT.

| | With KBD | Without KBD | Wald Chi-Square | *P* |
|---|---|---|---|---|
| Pecking$^{OFT}$ | 1.64 ± 0.33 | 1.09 ± 0.24 | 3.78 | 0.052 |
| Crossing the central zone $^{OFT}$ | 1.13 ± 0.18 | 1.41 ± 0.21 | 5.81 | 0.02 |
| LLAS (s)$^{SRT}$ | 17.73 ± 3.41 | 31.76 ± 5.94 | 6.62 | 0.01 |

*KBD, keel bone damage; OFT, open-field test; SRT, social reinstatement test; LLAS, latency to leave the start box.*

In contrast, we were unable to confirm that bone condition was associated with either physiological stress (measured as feather CORT concentration) or body weight.

In the OFT, the individual behavior was determined by different quantities of fear behavior (as number of times the target behavior was observed; **Table 2**) and was inconsistent with time, however, associated with bone condition at the peak of laying period (at 29 wa). This means that an individual used a strategy on an *ad hoc* basis based on how good/bad its keel health was. The behavior displayed seems to depend also on an individual life stage need (41) or may result from an increased willingness to move from the start box or from a habituation effect (42, 43), since being in lay led to an increase in the number of our hens leaving the start box and reaching the central zone. This change might also depend on age or early experience because fearful shyness occurs among the young of most mammalian species (44), which could also be true for the bird. Considering the impact of affective states on bone damage, our results show that fear responses categorized from calm to highly fearful, a high psychological state of fear at 17 and 18 wa, but also high physiological stress assessed by feather CORT concentration (at 33 and 58 wa) or low body weight (at 47 and 58 wa) were not associated with less bone damage at 29 wa, which is contrary to our expectations. As reviewed by Harlander-Matauschek et al. (30), it could be that with regard to KB fractures, fearful hens may be more likely to panic and thus collide with pen furnishing, leading them to develop worse bone health. Our current results are incongruent with expectations based on this literature. However, no study has been reported to clearly investigate and demonstrate the link between underlying fearfulness and bone damage in animals. Still, one possible reason for not confirming our first hypothesis is the exposure of our hens to only two acute fear-induced situations before the bone damage reached the prevalence of half of the hens. One could also argue that the responses recorded are unrepresentative of actual fearfulness because they were not investigated in the hen's home pen.

Considering the consequences of bone damage, hens with bone damage at the peak of the laying period (29 wa) had similar feather CORT concentrations at 33 and 58 wa and body weight at 47 and 58 wa. When exposed to open field-induced fear situation at 29 wa, these hens were recorded as having moved less often between zones in the test arena. According to the assumptions of the OFT (45), this can be a measure of worse locomotor behavior in chickens. Since these hens also showed more pecking on the ground and the wall, it is less likely that this behavior is a sign of exploratory pecking. Given the presence of escape attempts (recorded as fear responses and labeled as highly fearful animals), these responses suggest that they perceived the situation as more threatening or fearful. These findings also suggest that fearfulness, bone damage, and pecking behavior are related, a link proposed previously with injurious pecking behavior (23).

In many species, fear level has been negatively correlated with social motivation [birds (46), pigs (47), horses (48)], and this has been linked to high physiological stress responses [birds (49), pigs (50), humans (4)]; however, our results contradict these links. In the social situation at 42 wa, the responses of hens with bone damage suggest improved sociality with animals leaving the start box. According to models of motivation (51), these responses may indicate that individuals had a higher motivation to explore a subject/environment or a lower fear or anxiety level in a social context. However, this result may also be interpreted as a sign of fast decision-making (52) or boldness (53) with active seeking to escape and social reinstatement (54) or sensitization (55). When coupled with the argument of Mills et al. (46) that social motivation predominates over the fear response in individuals with a high tendency of making social contact, hens with bone damage imply to develop a different biological sensitivity to the social context (44) compared to hens without damage. Whatever the reason, it appears that bone damage, which potentially causes pain, particularly when bone is broken (5, 9), leads to different fear- and social-related psychological stress in birds, but not necessarily stress-induced CORT. It must be emphasized that this interpretation is based on an analysis where fractures and deviations were considered in a single variable due to the method applied, which is most practical from a commercial perspective but is not reliable enough to detect all differences in damage nor the time of a fracture. Evidence suggests that bone fractures have a negative impact on self-esteem, body image, and mood in humans (2) as well as negative affective states in laying hens (24).

It has also been documented that humans (2, 56) and animals [dogs (57), chicken (58), mice (59)] can encounter problems with bone condition due to body weight. It remains unclear why in species, such as birds, the KB is not under the influence of body weight, although it is in conjunction with the studies of Nasr et al. (13, 19) using a highly productive Lohmann Brown laying strain. One possible explanation for not detecting its influence could be the low variation in body weight found in this study.

It also remains unsettled why after the ossification is completed, at 45 wa, all hens ended up with a bone deviation or fracture, which seems to be a general phenomenon (7, 60). They experienced the same level of feather CORT deposition, regardless of the presence of damage. It is known that stress hormones like CORT in birds or cortisol in humans and other mammalian animals are important for the body's ability to respond to stress and injury. They are known to have complex effects on the skeleton, with small amounts needed for normal bone development but large amounts inhibiting bone growth (61). The finding of increased cumulative CORT deposition with age was similarly established in another recent chicken study (62).

This in conjunction with the evidence in humans that prolonged treatment with glucocorticoids can produce osteoporosis (63), allowing us to speculate that our hens may produce osteoporosis characterized by a decrease in bone mass that may thus be related to KBD. In humans, it is reported (2) that bone adapts to stress with age, although its ability depends on both genetic factors and lifestyle, a phenomenon not yet proven in chickens. Nevertheless, knowing that the KB is typically reduced or absent in flightless birds (64, 65), likely as its main function is to provide adequate leverage for flight, and by assuming today's chickens are poor flyers (66) and very good egg producers, one can argue that the skeletal adaptation has changed with selection for high egg yield, increasing the frequency of KB breakage. Greater understanding of physiological and psychological stress-related relationships may help to reduce levels of damage and severity in modern chickens.

## CONCLUSIONS

Psychosocial factors such as fear-induced pecking and locomotor reactions and sociality revealed an association with the development of an adverse bone condition in hens. Knowing that an individual's success with surviving and reproducing depends critically on its behavior, in the present work, we propose that hens with poor bone condition may experience psychological consequences from KBD but also that fear- and social-related psychological stress may be a potential predictor of bone damage.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

All procedures with animals (experimental protocols and methods) were performed according to the legislation on animal experimentation in Slovenia and were approved by animal-welfare body at the Department of Animal Science, that is a member of Ethical Committee of the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection (UVHVVR) and were in accordance with the principals 3Rs.

## AUTHOR CONTRIBUTIONS

NR contributed to the conceptualization, methodology, formal analysis, investigation, and writing the original draft. MZ contributed to the conceptualization, methodology, visualization, resources, writing, review and editing, and supervision. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was a part of the Slovene national project V4-1604 granted by the Slovenian Research Agency and Ministry of Agriculture, Forestry and Food (MKGP) Slovenia.

## ACKNOWLEDGMENTS

The work considers the research topic covered within COST Action CA15224. We thank Ivan Dimitrov from the Agricultural Institute at Stara Zagora, Bulgaria, who visited our research center and was involved in the initial stage of study preparation. We are grateful to Dušan Terčič for technical assistance and logistical support, Nataša Debeljak for practical support, Špela Malovrh for initial statistical advice, and Nina Cebulj Kadunc from the Veterinary Faculty of the University of Ljubljana who provided laboratory facilities for corticosterone analyses. We wish to express our great gratitude to Sandra Edwards and Murray Bales for help with the English and for their comments on the manuscript. The research is part of the Master thesis of NR at the Department of Animal Science, Biotechnical Faculty of the University of Ljubljana, Slovenia.

## REFERENCES

1. Brenneman RE. Osteoporosis the silent disease. Prevention treatment of fragility fractures in a structured program. *JLGH.* (2016) 11:112–6. Available online at: http://www.jlgh.org/JLGH/media/Journal-LGH-Media-Library/Past%20Issues/Volume%2011%20-%20Issue%204/Osteoporosis-the-Silent-Disease.pdf
2. U.S. Department of Health Human Services, Office of the Surgeon General. *Bone Health Osteoporosis: A Report of the Surgeon General.* Rockville, MD: Superintendent of Documents, U.S. Government Printing Office (2004).
3. Vellas BJ, Wayne SJ, Romero LJ, Baumgartner RN, Garry PJ. Fear of falling and restriction of mobility in elderly fallers. *Age Ageing.* (1997) 26:189–93. doi: 10.1093/ageing/26.3.189
4. Nees F, Witt SH, Flor H. Neurogenetic approaches to stress and fear in humans as pathophysiological mechanisms for posttraumatic stress disorder. *Biol Psychiatry.* (2018) 83:810–20. doi: 10.1016/j.biopsych.2017.12.015
5. Lay, Jr DC, Fulton RM, Hester PY, Karcher DM, Kjaer JB, et al. Hen welfare in different housing systems. *Poult Sci.* (2011) 90:278–94. doi: 10.3382/ps.2010-00962
6. Heerkens JL, Delezie E, Rodenburg TB, Kempen I, Zoons J, Ampe B, et al. Risk factors associated with keel bone and foot pad disorders in laying hens housed in aviary systems. *Poult Sci.* (2015) 95:482–8. doi: 10.3382/ps/pev339
7. Stratmann A, Frohlich EKF, Harlander-Matausche A, Schrader L, Toscano MJ, Wurbel H, et al. Soft perches in an aviary system reduce incidence of keel bone damage in laying hens. *PLoS ONE.* (2015) 10:e0122568. doi: 10.1371/journal.pone.0122568
8. Toscano MJ, Booth F, Wilkins LJ, Avery NC, Brown SB, Richards G, et al. The effects of long (c20/22) and short (c18) chain omega-3 fatty acids on keel bone fractures, bone biomechanics, behavior, egg production in free range laying hens. *Poult Sci.* (2015) 94:823–35. doi: 10.3382/ps/pev048
9. Riber AB, Casey-Trott TM, Herskin MS. The influence of keel bone damage on welfare of laying hens. *Front Vet Sci.* (2018) 5:6. doi: 10.3389/fvets.2018.00006
10. Toscano MJ, Dunn IC, Christensen JP, Petow S, Kittelsen K, Ulrich R. Explanations for keel bone fractures in laying hens: are there explanations in addition to elevated egg production? *Poult Sci.* (2020) 99:4183–94. doi: 10.1016/j.psj.2020.05.035

11. Bain MM, Nys Y, Dunn IC. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? *Br Poult Sci.* (2016) 57:330–8. doi: 10.1080/00071668.2016.1161727
12. Gebhardt-Henrich SG, Frohlich EK. Early onset of laying and bumblefoot favor keel bone fractures. *Animals.* (2015) 5:1192–206. doi: 10.3390/ani5040406
13. Nasr MA, Nicol CJ, Murrell JC. Do laying hens with keel bone fractures experience pain? *PLoS ONE.* (2012) 7:e42420. doi: 10.1371/journal.pone.0042420
14. Darwin C. The variation of animals plants under domestication. In: Van Wyhe J, editor. *The Complete Work of Charles Darwin Online*. Darwin online, London: John Murray (1868). Available online at: http://darwin-online.org.uk/content/frameset?itemID=F878.1&viewtype=text&pageseq=1 (accessed April 10, 2019).
15. Warren DE. Physiological genetic studies of crooked keels in chickens. *Kansas Agricultural Experiment Station Technical Bulletin*. (1937). Available online at: https://www.ksre.k-state.edu/historicpublications/pubs/STB044.PDF (accessed December 15, 2018).
16. Winter AR, Funk EM. *Poultry Science Practice*. Philadelphia, PA: Lippincott, JB (1941).
17. Buckner GD, Insko WM Jr, Henry AH, Wachs EF. Rate of growth and calcification of the sternum of male and female new hampshire chickens. *Poult Sci.* (1948) 27:430–33. doi: 10.3382/ps.0270430
18. Casey-Trott T. *Opportunities for exercise during pullet rearing: effects on bone health and keel bone damage in laying hens*. (Dissertation), University of Guelph, Guelph, ON, Canada (2016).
19. Nasr MAF, Murrell J, Nicol CJ. The effect of keel fractures on egg production, feed and water consumption in individual laying hens. *Br Poult Sci.* (2013) 54:165–70. doi: 10.1080/00071668.2013.767437
20. Fraisse F, Cockrem JF. Corticosterone fear behaviour in white and brown caged laying hens. *Br Poult Sci.* (2006) 47:110–9. doi: 10.1080/00071660600610534
21. Ghareeb K, Niebuhr K, Awad WA, Waiblinger S, Troxler J. Stability of fear and sociality in two strains of laying hens. *Br Poult Sci.* (2008) 49:502–8. doi: 10.1080/00071660802290390
22. Edwards LE, Coleman GJ, Hemsworth PH. Close human presence reduces avoidance behaviour in commercial caged laying hens to an approaching human. *Anim Prod Sci.* (2013) 53:1276–82. doi: 10.1071/AN12342
23. Riber AB, Hinrichsen LK. Welfare consequences of omitting beak trimming in barn layers. *Front Vet Sci.* (2017) 4:222. doi: 10.3389/fvets.2017.00222
24. Armstrong EA, Rufener C, Toscano MJ, Eastham JE, Guy JH, Sandilands V, et al. Keel bone fractures induce a depressive-like state in laying hens. *Sci Rep.* (2020) 10:3007. doi: 10.1038/s41598-020-59940-1
25. Rauw WM, Johnson AK, Gomez-Raya L, Dekkers JCM. A hypothesis and review of the relationship between selection for improved production efficiency, coping behavior and domestication. *Front Genet.* (2017) 8:134. doi: 10.3389/fgene.2017.00134
26. de Haas EN, Kops MS, Bolhuis JE, Groothuis TG, Ellen ED, Rodenburg TB. The relation between fearfulness in young and stress-response in adult laying hens, on individual and group level. *Physiol Behav.* (2012) 107:433–9. doi: 10.1016/j.physbeh.2012.08.002
27. Bortolotti GR, Marchant TA, Blas J, German T. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. *Funct Ecol.* (2008) 22:494–500. doi: 10.1111/j.1365-2435.2008.01387.x
28. Šalehar A, Kompan D, Žan Lotrič M, Kovač M, Holcman A, Cepon M, et al. *Razvoj Pasem Domačih Živali v Sloveniji: Prvotne, Izgubljene in Pretopljene Pasme*. 2nd ed. Biotehniška fakulteta: Oddelek za zootehniko, Rodica, Slovenia (2014). 232p.
29. Jones RB. Fear adaptability in poultry: insights, implications imperatives. *World Poult Sci J.* (1996) 52:131–74. doi: 10.1079/WPS19960013
30. Harlander-Matauschek A, Rodenburg T, Sandilands V, Tobalske B, Toscano M. Causes of keel bone damage their solutions in laying hens. *World Poult Sci J.* (2015) 71:461–72. doi: 10.1017/S0043933915002135
31. Doherty WJ, DeRome ME, McCarthy MB, Gronowicz GA. The effect of corticosterone on osteoblast expression of beta 1 integrins. *J Bone Joint Surg Am.* (1995) 77:396–404. doi: 10.2106/00004623-199503000-00009
32. Genné-Bacon EA, Trinko JR, DiLeone RJ. Innate fear-induced weight regulation in the C57BL/6J mouse. *Front Behav Neurosci.* (2016) 10:132. doi: 10.3389/fnbeh.2016.00132
33. Romero LM, Fairhurst GD. Measuring corticosterone in feathers: strengths, limitations, suggestions for the future. *Comp Biochem Physiol A Mol Integr Physiol.* (2016) 202:112–22. doi: 10.1016/j.cbpa.2016.05.002
34. Casey-Trott T, Heerkens JLT, Petrik M, Regmi P, Schrader L, Toscano MJ, et al. Methods for assessment of keel bone damage in poultry. *Poult Sci.* (2015) 94:2339–50. doi: 10.3382/ps/pev223
35. Gebhardt-Henrich SG, Rufener C, Stratmann A. Improving intra- inter-observer repeatability and accuracy of keel bone assessment by training with radiographs. *Poult Sci.* (2019) 98:5234–40. doi: 10.3382/ps/pez410
36. de Haas EN, Lee C, Hernandez EC, Naguib M, Rodenburg BT. Individual differences in personality in laying hens are related to learning a colour cue association. *Behav Processes.* (2017) 134:37–42. doi: 10.1016/j.beproc.2016.11.001
37. Agnvall B, Jöngren M, Strandberg E, Jensen P. Heritability genetic correlations of fear-related behaviour in red junglefowl–possible implications for early domestication. *PLoS ONE.* (2012) 7:e35162. doi: 10.1371/journal.pone.0035162
38. Dawkins MS. Distance, social recognition in hens: implications for the use of photographs as social stimuli. *Behaviour.* (1996) 133:663–80. doi: 10.1163/156853996X00413
39. Jahn-Eimermacher A, Lasarzik I, Raber J. Statistical analysis of latency outcomes in behavioral experiments. *Behav Brain Res.* (2011) 221:271–5. doi: 10.1016/j.bbr.2011.03.007
40. Nakagawa S, Schielzeth H. Repeatability for gaussian and non-gaussian data: a practical guide for biologists. *Biol Rev Camb Philos Soc.* (2010) 85:935–56. doi: 10.1111/j.1469-185X.2010.00141.x
41. Weeks CA, Nicol CJ. Behavioural needs priorities preferences of laying hens. *Worlds Poult Sci J.* (2006) 62:296–307. doi: 10.1079/WPS200598
42. Geverink NA, Kappers A, Burgwal van de JA, Lambooij E, Blokhuis HJ, Wiegant VM. Effects of regular moving and handling on the behavioural and physiological consequences to preslaughter treatment and consequences for subsequent meat quality. *J Anim Sci.* (1998) 76:2080–5. doi: 10.2527/1998.7682080x
43. Forkman B, Boissy A, Meunier-Salaün MC, Canali E, Jones RB. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. *Physiol Behav.* (2007) 92:340–74. doi: 10.1016/j.physbeh.2007.03.016
44. Buss AH. A theory of shyness. In: Jones WH, Cheek JM, Briggs SR, editors. *Shyness. Perspectives on Research and Treatment. (Emotions, Personality, Psychotherapy)*. New York, NY: Springer Science+Business Media, Boston. (1986). p. 39–46. doi: 10.1007/978-1-4899-0525-3_4
45. Jones RB, Carcmichael N. Open-field behavior in domestic chicks tested individually or in pairs: differential effects of painted lines delineating subdivisions of the floor. *Behav Res Methods Instrum Comput.* (1997) 29:396–400. doi: 10.3758/BF03200593
46. Mills DA, Jones BR, Faure MJ. Species specificity of social reinstatement in Japanese quail coturnix japonica genetically selected for high or low levels of social reinstatement behaviour. *Behav Processes.* (1995) 34:13–22. doi: 10.1016/0376-6357(94)00044-H
47. Zebunke M, Puppe B, Langbein J. Effects of cognitive enrichment on behavioural and physiological reactions of pigs. *Physiol Behav.* (2013) 118:70–9. doi: 10.1016/j.physbeh.2013.05.005
48. Rørvang MV, Christensen JW. Attenuation of fear through social transmission in groups of same and differently aged horses. *Appl Anim Behav Sci.* (2018) 209:41–6. doi: 10.1016/j.applanim.2018.10.003
49. Mills AD, Jones RB, Faure JM, Williams JB. Responses to isolation in Japanese quail genetically selected for high or low sociality. *Physiol Behav.* (1993) 53:183–9. doi: 10.1016/0031-9384(93)90029-F
50. Zupan M, Zanella AJ. Peripheral regulation of stress and fear responses in pigs from tail-biting pens. *R Bras Zootec.* (2017) 46:33–8. doi: 10.1590/s1806-92902017000100006
51. Jensen P, Toates FM. Who needs 'behavioural needs'? Motivational aspects of the needs of animals. *Appl Anim Behav Sci.* (1993) 37:161–81. doi: 10.1016/0168-1591(93)90108-2

52. Koolhaas JM. Coping style immunity in animals: making sense of individual variation. *Brain Behav Immun.* (2008) 22:662–7. doi: 10.1016/j.bbi.2007.11.006
53. Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, et al. Coping styles in animals: current status in behavior and stress-physiology. *Neurosci Biobehav Rev.* (1999) 23:925–35. doi: 10.1016/S0149-7634(99)00026-3
54. de Haas EN, Lee C, Rodenburg TB. Learning judgment can be affected by predisposed fearfulness in laying hens. *Front Vet Sci.* (2017) 4:113. doi: 10.3389/fvets.2017.00113
55. Ordiz A, Moen GK, Sæbø S, Stenset N, Swenson JE, Støen OG. Habituation sensitization, or consistent behavioral responses? Brown bear responses after repeated approaches by humans on foot. *Biol Conserv.* (2019) 232:228–37. doi: 10.1016/j.biocon.2019.01.016
56. Mitchell JE, Crow S. Medical complications of anorexia nervosa and bulimia nervosa. *Curr Opin Psychiatry.* (2006) 19:438–43. doi: 10.1097/01.yco.0000228768.79097.3e
57. Czirják TZ, Chereji A. Canine obesity – a major problem of pet dogs. *Ecotox Zooteh Ind Alim.* (2008) 7:361–6. Available online at: http://protmed.uoradea.ro/facultate/anale/ecotox_zooteh_ind_alim/2008/Czirjak%20Zsolt.pdf
58. Stojcic MD, Bessei W. The effect of locomotor activity and weight load on bone problems in fast and slow growing chickens. *Arch Geflügelkund.* (2009) 73:242–9. Available online at: https://www.european-poultry-science.com/artikel.dll/m08-25mk_MTE2NzkxNg.PDF?UID=904E30112A9F988C3E86AD9D4686BEB7EB28D0DA38C28F
59. Cao JJ. Effects of obesity on bone metabolism. *J Orthop Surg Res.* (2011) 6:30. doi: 10.1186/1749-799X-6-30
60. Toscano MJ, Booth F, Richards G, Brown SN, Karcher DM, Tarlton JF. Modeling collisions in laying hens as a tool to identify causative factors for keel bone fractures and means to reduce their occurrence and severity. *PLoS ONE.* (2018) 13:e0200025. doi: 10.1371/journal.pone.0200025
61. Canalis E, Delany AM. Mechanisms of glucocorticoid action in bone. *Ann N Y Acad Sci.* (2002) 966:73–81. doi: 10.1111/j.1749-6632.2002.tb04204.x
62. Nordquist RE, Zeinstra EC, Dougherty A, Riber AB. Effects of dark brooder rearing and age on hypothalamic vasotocin and feather corticosterone levels in laying hens. *Front Vet Sci.* (2020) 7:19. doi: 10.3389/fvets.2020.00019
63. Mazziotti G, Angeli A, Bilezikian JP, Canalis E, Giustina A. Glucocorticoid-induced osteoporosis: an update. *Trends Endocrinol Metab.* (2006) 17:144–9. doi: 10.1016/j.tem.2006.03.009
64. Gill FB. *Ornithology*. 2nd ed. Portland, OR: Freeman WH Company (1994).
65. Harshman J, Braun EL, Braun MJ, Edward L, Huddleston CJ, Bowie RCK, et al. Phylogenomic evidence for multiple losses of flight in ratite birds. *Proc Natl Acad Sci USA.* (2008) 105:13462–7. doi: 10.1073/pnas.0803242105
66. Ekesbo I. *Farm Animal Behaviour: Characteristics for Assessment of Health Welfare*. Wallingford; Cambridge: CABI (2011). doi: 10.1079/9781845937706.0000


**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Advantages of publishing in Frontiers

**OPEN ACCESS**
Articles are free to read for greatest visibility and readership

**FAST PUBLICATION**
Around 90 days from submission to decision

**HIGH QUALITY PEER-REVIEW**
Rigorous, collaborative, and constructive peer-review

**TRANSPARENT PEER-REVIEW**
Editors and reviewers acknowledged by name on published articles

**Frontiers**
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

**Visit us:** www.frontiersin.org
**Contact us:** frontiersin.org/about/contact

**REPRODUCIBILITY OF RESEARCH**
Support open data and methods to enhance research reproducibility

**DIGITAL PUBLISHING**
Articles designed for optimal readership across devices

**FOLLOW US**
@frontiersin

**IMPACT METRICS**
Advanced article metrics track visibility across digital media

**EXTENSIVE PROMOTION**
Marketing and promotion of impactful research

**LOOP RESEARCH NETWORK**
Our network increases your article's readership