



Daily behavioral patterns of laying hens kept in a multi-tier system across different microclimatic conditions

Mattia Pravato ^{a,} , Francesco Bordignon ^{a,} , Gerolamo Xiccato ^{a,} , Giulia Zorzi ^a,
Andrea Pezzuolo ^{a,} , Francesco Marinello ^{b,} , Angela Trocino ^{a, c, *}

^a Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

^b Department of Land, Environment, Agriculture and Forestry (TESAF), University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

^c Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

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ABSTRACT

The present study compared the daily behavioral pattern of laying hens in a multi-tier system at different months representing different microclimatic conditions. The behavior at the different levels (ground, first, second and third tier) of 450 laying hens in two pens was video recorded three days per month and then scored every 15 minutes. On the ground, the rate of pecking hens was lower in January compared to March and June, while the rate of preening and ground scratching hens was higher ($P < 0.001$); in March, both the rate of dustbathing hens ($P = 0.004$) and the occurrence of piling ($P < 0.001$) showed a peak. Across the three tiers, the rates of perching ($P < 0.001$) and preening hens ($P < 0.001$) were higher in January compared to March and June, while the rate of hens pecking the tier was lower ($P < 0.001$). Finally, the rate of hens resting on the tiers was higher in January compared to March ($P = 0.01$) with intermediate values in June. Overall, laying hens exhibited a functional space use of the multi-tier system, expressing species-specific behaviors according to available resources and equipment and consistent daily behavioral pattern across different microclimatic conditions, despite showing month-specific differences at certain hours. Some changes in the behavioral patterns at the different tiers across the different microclimatic conditions were possibly dependent on the corresponding different number of animals, i.e. a different vertical distribution of animals depending on microclimatic conditions.

1. Introduction

Poultry production has been facing serious challenges in effectively managing the welfare of laying hens in the context of a progressive shift toward housing and management systems alternative to cages. In fact, while the transition from conventional to enriched cages does not substantially change or improve the ethogram of laying hens (Onbaşlar et al., 2022), available studies show that hens housed in cage-free systems exhibit a wide behavioral pattern (Xu et al., 2022). Nevertheless, under all production systems hens are sensitive to different welfare consequences (i.e. negative changes in welfare resulting from one or more risk factors) which can lead to increased aggression, fear responses, injurious pecking and piling behavior (EFSA AHAW Panel (EFSA Panel on Animal Health and Animal Welfare) et al., 2023). The

experience of stress and negative emotional state has also been associated with a disruption of daily behavioral pattern, further compromising welfare (Mench, 2009). Among alternative cage-free systems, which include floor and multi-tiers systems besides systems with outdoor access, modern aviary systems are associated with increased complexity for management and increased size of commercial flocks. Under these conditions, advanced technologies for controlling and monitoring environmental conditions are key tools for maintaining animal welfare while also supporting productivity goals (David et al., 2015a, 2015b; Rowe et al., 2019). In fact, continuous monitoring can ensure early detection of welfare and health issues, guarantee appropriate living conditions, and enable targeted interventions (Bordignon et al., 2025b) which gets even more importance under the challenging global conditions of climate change. Suitable ranges must be guaranteed for

Angela Trocino. Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE). University of Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy. Tel. +39 049 8272583

* Corresponding author.

E-mail address: angela.trocino@unipd.it (A. Trocino).

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parameters, such as temperature, ammonia, carbon dioxide, and particulate matter, within the rearing environment (David et al., 2015a, 2015b; Bordignon et al., 2025a). Temperature deviations from optimal ranges are known to affect feed intake and efficiency and, thus, production performance, besides increasing susceptibility to different diseases (Ghoname et al., 2022). Then, relative humidity, interacting also with temperature, regulates heat dissipation in animals and litter quality, where present, which in turn affect sensitivity of chickens to several diseases, from skin disorders (Mou et al., 2025) to respiratory diseases (Wang et al., 2022). Finally, impaired air quality, as for increased ammonia, carbon dioxide and dust, can challenge health for immune competence and respiratory disorders (Oliveira et al., 2021). Variations in microclimatic conditions are also associated with behavioral changes, where animals start to adopt behavioral strategies for mitigating discomfort (such as lying laterally and inactive) at the expense of other functional behaviors (such as walking and running) (Branco et al., 2020). On the other hand, the effective management of microclimatic conditions depends on production systems, available facilities, and equipment (Küçüktopcu et al., 2022). Indeed, while optimal ranges of microclimatic parameters for physiological homeostasis and, consequently, production performance are rather well defined, the sensitivity of poultry to different microclimatic conditions are known to change with animal age, category, and genotype (Mignon-Grasteau et al., 2015). Then, while average conditions within the barn can be consistent with animal requirements, microclimate at different levels of housing systems can change, which in turn can affect space use and distribution of animals within the system (Bordignon et al., 2025a). In this context, in a previous paper (Bordignon et al., 2025a), we reported how environmental micro-climatic conditions during different months change at different heights within a multi-tier system and relationships with the spatial distribution of two genotypes of laying hens, White and Brown ones, between 31 and 52 weeks of age. Accordingly, we hypothesized that changes in animal distribution and use of resources because of the differences in microclimatic conditions could have also affected their daily behavioral pattern. Thus, this paper complements the previous one by presenting further results related to the same study and reporting the effects of the microclimatic conditions at the different months on the behavioral repertoire and the daily behavioral pattern of the animals of the same trial (Bordignon et al., 2025a).

2. Material and methods

2.1. Experimental facility, animals and experimental arrangement

The trial was carried out at the farm “L. Toniolo” of the University of Padova, using an experimental aviary (2.50 m wide × 19.52 m long × 2.83 m high) consisting of three tiers and a litter area on the ground. Two corridors were available for the hens along the two sides of the aviary, so the ground space was 6.01 m wide × 19.52 m long. The aviary was divided into eight pens (2.50 m wide × 2.44 m long × 2.83 m high), each hosting 225 animals at housing time (18 weeks of age) at 9 hens/m² (including the tiers). Each pen was equipped with four collective nests (nest area: 0.51 m²). A programmable logic controller (Officine Faccio & C. Spa, Campo San Martino, Padova, Italy) managed feeding, drinking, lighting, and ventilation inside the system. The first tier of the aviary (at a height of 54 cm) was equipped with automatic feeders, nipple drinkers, feeding perches and external perches (length 120 cm). The second tier (136 cm of height) was equipped with collective nests and nipple drinkers. Finally, the third uppermost tier (220 cm of height) had only automatic feeders, continuous perches, feeding perches, and uppermost perches along the whole length of the pen (Supplementary Fig. S1).

A total of 1800 hens was delivered to the experimental farm by an authorized truck and housed in the 8 pens of the aviary. Half of the hens were brown-feathered (initial live weight: 1304 g ± 129) and half of the hens white-feathered (live weight: 1172 g ± 90.8) (Novogen S.A.S.,

Pledran, France); white and brown hens were housed in separate pens (4 pens per genotype).

For the specific purposes of this study, two pens were monitored, i.e. one pen of white hens and one pen of brown hens, from 31 to 52 weeks of age (average oviposition rate >90%). During this period, the hens were exposed to a 16-h light/8-h dark photoperiod (dawn starting at 4:00 and dusk starting at 20:00) and fed ad libitum the same commercial diet, based on maize and soybean meal, formulated to meet the nutritional requirements of laying hens (Novogen, 2025a, 2025b). The feed was automatically distributed four times a day (05:00, 08:00, 12:00, and 16:00 h).

As detailed by Bordignon et al. (2025a), two environmental sensors (N11-Air quality IBT-Systems; IBTSystems, Milano, Italy) were used to monitor a range of environmental variables at different sampling heights of the pens (ground, second tier and third tier) during three periods (January, March, and June). On average of the daily recordings, the following data were recorded in January: temperature 17.3 ± 1.36 °C; relative humidity 47.1 ± 6.41%; carbon dioxide 1201 ± 171 ppm; and ammonia 3.12 ± 1.67 ppm. In March, the environmental variables were: temperature 17.2 ± 1.46 °C; relative humidity 57.8 ± 4.49%; carbon dioxide 806 ± 163 ppm; and ammonia 2.27 ± 1.16 ppm. Finally, in June, the recorded values were: temperature 21.7 ± 1.58 °C; humidity 74.4 ± 9.47%; carbon dioxide 587 ± 158 ppm; and ammonia 0.75 ± 1.36 ppm. All environmental data collected by the environmental sensors during the months of January, March, and June and already published by Bordignon et al. (2025a) are reported in supplementary Table S1.

2.2. Video recordings, hen distribution, and behavioral evaluation

The behavior of the laying hens was video recorded in the two selected pens, i.e. one pen of white hens and one pen of brown hens, within the aviary three days per month from 4:00 to 21:00 on January, March, and June, when hens were 31, 37, and 50 weeks of age, respectively. To this purpose, a real-time video-recording system was used with a total of 48 cameras (Infrared mini-dome bullet 4 mp; resolution 1080 p) (HAC-HDW1220MP, Zhejiang Dahua Technology Co. Ltd., Hangzhou, China) and two full HD video-recorders (NVR2116HS-4KS2, Zhejiang Dahua Technology Co. Ltd.). The cameras were located to record the hens on four levels: hens on the ground (one camera per pen hanged at about 3 m of height); hens on the first and second tier of the aviary (one camera per pen hung at about 1.50 m of height); hens on the third tier of the aviary (one camera per pen hung at about 2.80 m of height and one camera per pen hung over the third tier).

Then, the number of hens performing predetermined behaviors from 4:00 until 21:00 (first observation hour 4:00-5:00; last observation hour 20:00-21:00 including the dusk/dark period) were scored by two trained people every 15 minutes by scanning 10 consecutive seconds of video (4 observations per hour × 4 levels × 17 hours × 2 pens × 3 days × 3 months = 4896 observations; 1224 per level). The number of hens performing different mutually exclusive behaviors at the start of each observation was scored (Table 1) (Thuy Diep et al., 2018; Trocino et al., 2020; Pravato et al., 2026). Then data were expressed as the rate of hens performing the behavior out of the total number of hens observed on the ground or on each tier at each observation interval. The events of piling and aggression were scored as presence/absence per observation interval and expressed as occurrence/number observation intervals × 100 per tier, month, and observation hour. Inter-observer agreement assessed on a subset of observations independently scored by both observers, using a two-way random-effects intraclass correlation coefficient for absolute agreement (ICC(2,1)), showed moderate to excellent reliability (0.636-0.969) (Koo and Li, 2016).

2.3. Statistical analysis

Data related to the rate of animals performing a behavior in each pen

Table 1

Ethogram used for behavioral evaluation of white and brown laying hens (modified from Sokolowicz et al., 2020; Pravato et al., 2026).

Behavior	Description
Tier pecking	Behavior performed by a single individual that corresponds to scratching/digging the surface (not ground) with the claws of the hind limbs and subsequently pecking the surface in the area where the scratching action occurred.
Tier resting	Behavior performed by a single individual that corresponds to crouch on the tiers of the aviary.
Scratching on the ground	Behavior performed by a single individual that consists of scratching/digging the ground/litter surface with the claws of the hind limbs with the aim of moving a quantity of litter material to search for and expose food.
Pecking on the ground	Behavior performed by a single individual which consists of tapping the ground with its beak, with the aim of eating any food present on the ground.
Dustbathing	Behavior performed by a single individual which consists of carrying out a sequence of specific movements with the hind limbs, wings, neck, head and beak with the aim of covering the plumage and skin with litter material.
Perching	Behavior performed by a single individual which consists of perching, therefore positioning itself on a perch, and maintaining the position with the aim of resting or sleeping.
Preening	Behavior performed by a single individual that consists of using the beak to arrange, smooth and clean the feathers of the entire body; all this is facilitated by a secretion produced by the uropygial glands that the individual collects and distributes over the entire body to create a condition of impermeability.
Piling	Behavior carried out by a group of individuals which consists in their massing in a very restricted area of the aviary for a more or less long period, thus creating an area with a very high density of individuals.
Aggression	Behavior performed by two individuals, generally an aggressive subject and the subject of the aggression. In this case, any voluntary contact in the head and neck areas and any chasing between individuals is considered.

(i.e. the percentage of hens performing the behavior out of visible hens after arcsine transformation) and the occurrence of aggression and piling events per scan were subjected to analysis of variance by using a generalized linear mixed model and the PROC GLIMMIX of SAS (SAS Institute, 2013), with position (tier, when relevant), month, observation hour, and their interactions as fixed effects. The pen and the observation day were considered as random effects, whereas the observation intervals per hour were considered as repeated measures defining the subject as the combination of pen, position (tier, when relevant), month, observation day, and observation hour. The GLIMMIX PROC used a normal distribution and link function identity for data related to the rate of animals performing a behavior (arcsine transformed); a binomial distribution and link function logit for data related to the occurrence of aggression and piling events; and a covariance

structure type “variance components”.

3. Results

On average, hens on the ground were especially engaged in pecking (68.1% of visible hens), followed by preening (18.8%), scratching (6.85%) and dustbathing (6.29%); then, the occurrence of piling and aggression was rather low (3.68% and 1.47% of observations) (Table 2). As for the effect of the months, the rate of hens pecking the ground (56.9% vs. 68.3% and 79.0% of visible hens; $P < 0.001$) was lower in January compared to March and June, while the rate of preening (26.8% vs. 17.3% and 12.4%; $P < 0.001$) and ground scratching hens (11.4% vs. 5.46% and 3.65%; $P < 0.001$) was higher. In March, both the rate of dustbathing hens (4.91%, 8.98%, and 4.99% in January, March, and June, respectively; $P = 0.004$) and the occurrence of piling (1.72%, 7.35%, and 1.96%; $P < 0.001$) showed a peak. Finally, the occurrence of aggression on the ground was higher in January compared to March and June (3.19% vs. 0.74% and 0.49%; $P < 0.001$) (Table 2).

Then, as for behaviors observed on the three tiers (Table 3), most hens were perching (on average 41.1% of visible hens), followed by pecking the tiers (26.0%), preening (22.7%), and resting (10.2%). On the first tier, the rate of perching hens was lower (31.1%) than on the second (35.2%) and third (57.0%) tiers ($P < 0.001$), while the rates of hens pecking the tier (33.1% vs. 28.5% vs. 16.3%; $P < 0.001$) and preening (26.8% vs. 19.6% and 21.7%; $P < 0.001$) were higher (Table 3). Moreover, the rate of resting hens decreased from the second tier to the first and third ones (16.7% vs. 9.00% vs. 4.95%; $P < 0.001$). As for the effect of the month, the rate of perching (42.1% and 44.3% vs. 36.9% of visible hens; $P < 0.001$) and preening hens (29.8% vs. 18.8% and 19.4%; $P < 0.001$) was higher in January compared to March and June, while the rate of hens pecking the tier was lower (16.6% vs. 28.2% vs. 33.2%; $P < 0.001$). Finally, the rate of hens resting on the tier was higher in January compared to March (11.5% vs. 8.71%; $P = 0.01$) with intermediate values in June (10.5%). The occurrence of aggression was also higher in January compared to March and June (1.23% vs. 0.16% and 0.16% of observations; $P < 0.001$).

As for changes according to the observation hour (Fig. 1), the rate of hens pecking the ground showed small changes, but a peak at 13:00 after which a progressive increase was observed until light switching off ($P < 0.001$) (Fig. 1a). The rate of preening hens was the highest at light switching on and decreased progressively throughout the day, reaching zero around the light switching off ($P < 0.001$) (Fig. 1b). The rate of dustbathing hens increased from 9:00 to 12:00 to decrease thereafter with the minimum value at 16:00 ($P < 0.001$) (Fig. 1c). Finally, piling started to occur around 9:00 to decrease after 17:00 ($P < 0.001$) (Fig. 1d).

As for behaviors on the tiers, the rate of hens pecking the tiers showed peaks during the early morning (06:00 and 08:00) and late

Table 2

Behaviors on the ground of the multi-tier aviary system at different months (January, March, June). Data are the average of three observation days (one per week) in each of three months (January, March, and June).

	Position (P)	Month (M)			RMSE	P-value		
		January	March	June		M	Hour ¹ (H)	M × H
No. observation interval ²	1224	408	408	408				
Hens performing exclusive behaviors/ visible hens in the observation interval								
Ground pecking, %	68.1	56.9 ^a	68.3 ^b	79.0 ^c	5.49	<0.001	<0.001	0.541
Preening, %	18.8	26.8 ^c	17.3 ^b	12.4 ^a	5.00	<0.001	<0.001	0.008
Ground scratching, %	6.85	11.4 ^b	5.46 ^a	3.65 ^a	2.63	<0.001	0.133	0.030
Dustbathing, %	6.29	4.91 ^a	8.98 ^b	4.99 ^a	2.86	0.004	<0.001	0.302
Occurrence per observation intervals								
Piling, %	3.68	1.72 ^a	7.35 ^b	1.96 ^a	-	<0.001	<0.001	<0.001
Aggression, %	1.47	3.19 ^b	0.74 ^a	0.49 ^a	-	<0.001	<0.001	<0.001

¹Hour, observation hour. ²Observation interval: 10 sec every 15 min per observation hour. Abbreviations: RMSE: Root mean square error. Data are expressed as LS means.

^{a,b,c}Values with different superscript letters significantly differ ($P < 0.05$).

Table 3
Behaviors at different tiers of the multi-tier aviary system at different months (January, March, June). Data are the average of three observation days (one per week) in each of three months (January, March, and June).

	Position (P)			Month (M)			RMSE	P-value					
	First tier	Second tier	Third tier	January	March	June		P	M	Hour ¹ (H)	P × M	M × H	P × H
No. observation interval ²	1224	1224	1224	1224	1224	1224							
Hens performing exclusive behaviors/ visible hens in the observation interval													
Tier pecking, %	33.1 ^c	28.5 ^b	16.3 ^a	16.6 ^a	28.2 ^b	33.2 ^c	4.28	<0.001	<0.001	<0.001	0.054	<0.001	<0.001
Perching, %	31.1 ^a	35.2 ^b	57.0 ^c	42.1 ^b	44.3 ^b	36.9 ^a	4.46	<0.001	<0.001	<0.001	0.085	<0.001	<0.001
Preening, %	26.8 ^b	19.6 ^a	21.7 ^a	29.8 ^b	18.8 ^a	19.4 ^a	4.00	<0.001	<0.001	<0.001	0.060	<0.001	<0.001
Tier resting, %	9.00 ^b	16.7 ^c	4.95 ^a	11.5 ^b	8.71 ^a	10.5 ^{ab}	3.11	<0.001	0.01	<0.001	0.002	<0.001	<0.001

¹Hour, observation hour. ²Observation interval: 10 sec every 15 min per observation hour. Abbreviations: RMSE: Root mean square error. Data are expressed as LS means.
^{a,b,c}Values with different superscript letters significantly differ ($P < 0.05$).

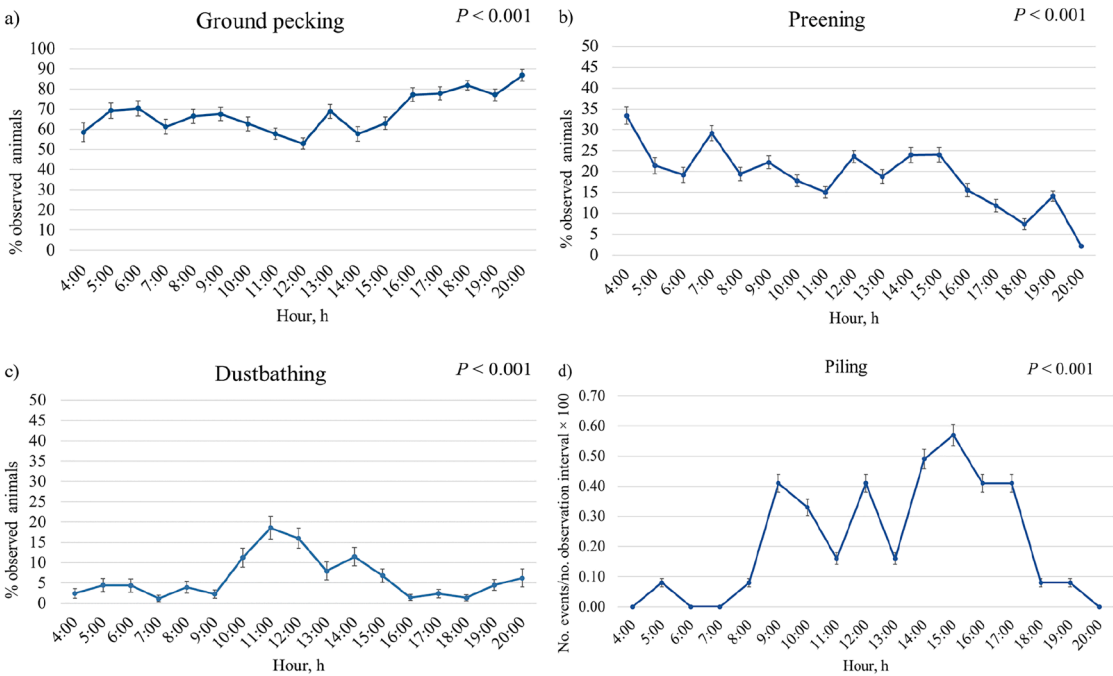


Fig. 1. Rate of laying hens (% of visible hens) performing pecking (a), preening (b), and dustbathing (c) and occurrence of piling (d) on the ground of the multi-tier aviary system at different hours of the day. Data are the average of three observation days (one per week) per three months (January, March, and June).

afternoon (16:00-17:00), whereas it markedly decreased from 18:00 onwards ($P < 0.001$) (Fig. 2a). The rate of perching hens mildly increased from early morning to midday, then it decreased during the afternoon until 16:00 to rise thereafter with the highest values (more than 90% of visible hens) recorded at 20:00 ($P < 0.001$) (Fig. 2b). On the other hand, the rate of preening hens was the highest (around 45% of visible hens) early in the morning and decreased during the day to reach the minimum at light switching off (Fig. 2c). The rate of hens resting on the tiers showed a fluctuating pattern throughout the day, being on average lower during early and midday hours (until 12:00), and higher, with some peaks, during afternoon ($P < 0.001$) (Fig. 2d).

Significant interactions were recorded between the months and the observation hours (Fig. 3 and Fig. 4). The effects of the interaction between the tier levels and the observation hours (Supplementary Fig. S2) and between the tier levels and the months (Supplementary Fig. S3) were statistically significant but showed uneven changes, not associated with any clear and/or relevant pattern.

4. Discussion

4.1. Behavioral pattern of laying hens across aviary levels and microclimatic conditions

The expression of a full behavioral repertoire with species-specific behaviors is universally recognized to be deeply linked to animal welfare. Indeed, under farming conditions, the transition to cage-free systems, both indoor and outdoor, is intended to provide hens with more opportunities for behavioral expression compared to conventional caged-housing, which has also been associated with reduced fear levels (Jeon et al., 2025). On the other hand, unsuitable space use and equipment can challenge the successful implementation of cage-free systems and can be affected by environmental microclimatic conditions. In fact, previous studies showed that the vertical distribution of the hens within the multi-tier aviary is significantly influenced by environmental conditions, with temperature and ventilation potentially playing a central role (David et al., 2015a). The previously published data related to the same trial of the present paper (Bordignon et al., 2025a) have highlighted the relevance of environmental vertical

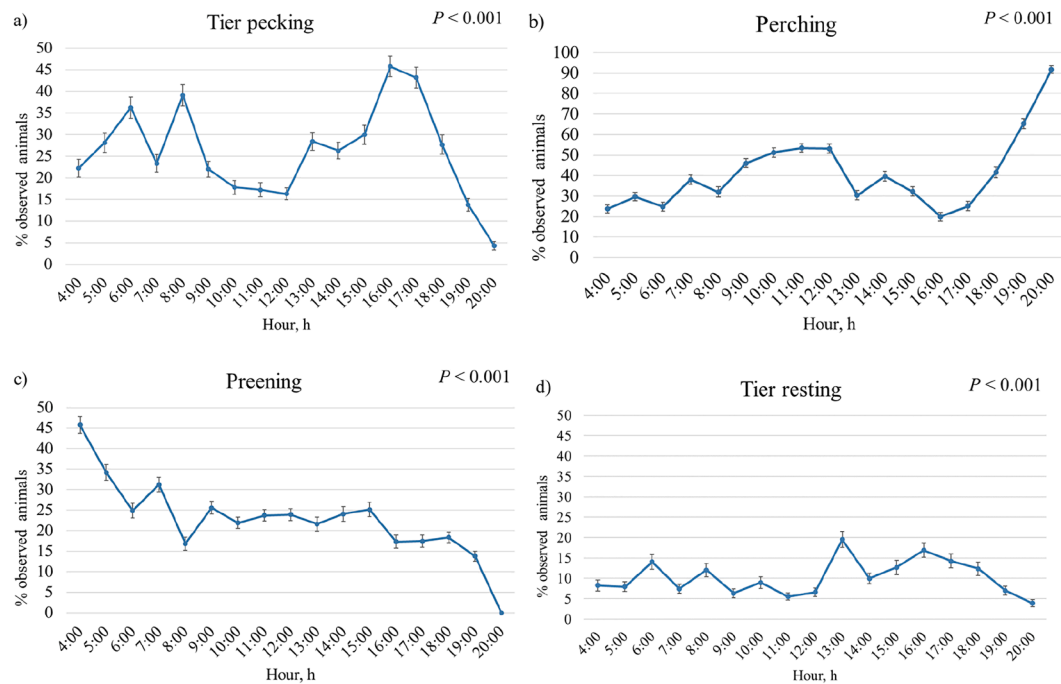


Fig. 2. Rate of laying hens (% of visible hens) performing pecking (a), perching (b), preening (c), and resting (d) on the tiers (average of the observations on the three tiers) of the multi-tier aviary system at different hours of the day. Data are the average of three observation days (one per week) per three months (January, March, and June).

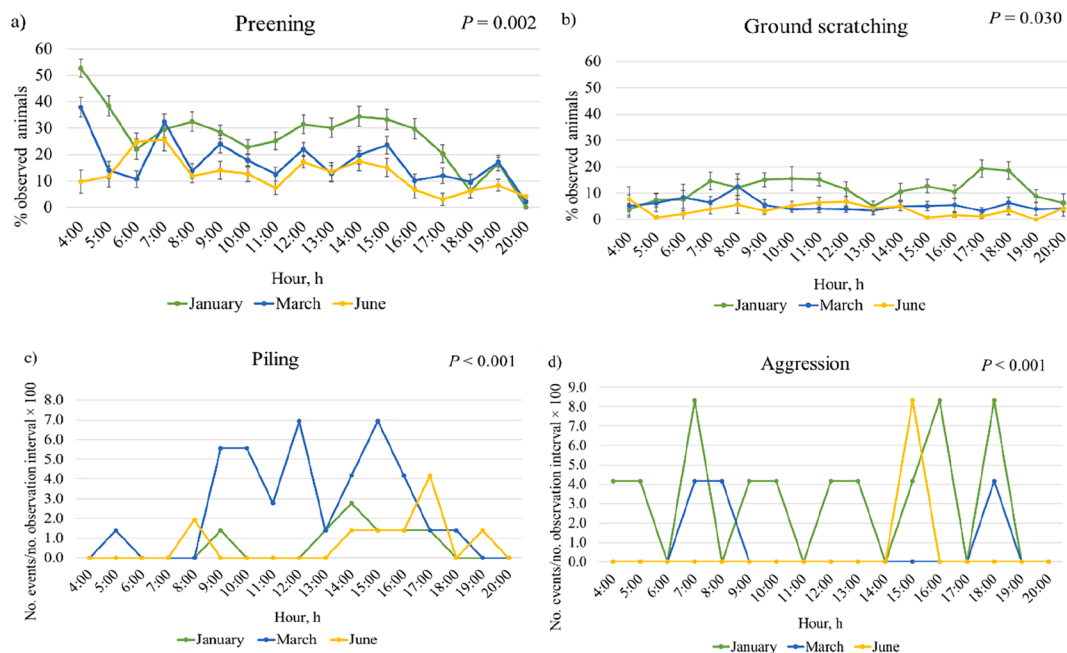


Fig. 3. Rate of laying hens (% of visible hens) performing preening (a), scratching (b), and occurrence of piling (c) and aggression (d) on the ground of the multi-tier aviary system at different hours of the day during January, March and June (significant interaction hour of the day $\times$ month). Data are the average three observation day (one per week) per three months (January, March, and June).

gradients within the aviary system and the role of microclimate on shaping spatial preferences of laying hens. On the other hand, the results presented in this paper focus on the role of the same factors in behaviors and daily patterns. Overall, we observed a functional use of the different areas in the tested multi-tier system, with most of the hens on the ground displaying exploration (i.e., scratching and pecking) and comfort behaviors (i.e., dustbathing). On the other hand, perching and preening, which are usually associated behaviors (Bist et al., 2023), were

especially observed on the upper tiers.

As for behaviors on the ground, the presence of large usable areas and access to litter in single and multi-tier systems allow pecking and especially scratching behaviors over the ground (Xu et al., 2022; Nutsugah et al., 2026), which are restrained in conventional cages (Lay et al., 2011). These behaviors represent the core component of foraging in laying hens; they are driven by intrinsic motivational mechanisms, rather than nutritional needs, and expressed even when feed is available

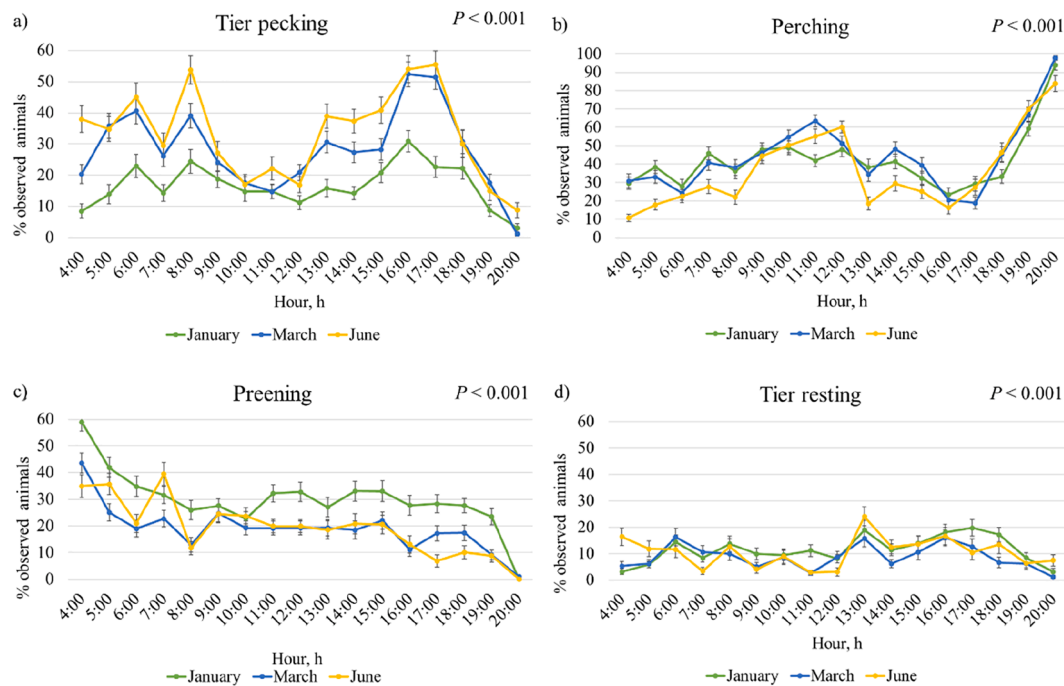


Fig. 4. Rate of laying hens (% of visible hens) performing pecking (a), perching (b), preening (c), and resting (d) on the tiers (average of the observations on the three tiers) of the multi-tier aviary system at different hours of the day during January, March and June (significant interaction hour of the day $\times$ month). Data are the average of three observations day (one per week) per three months (January, March, and June).

(Degrande et al., 2024). Importantly, when hens are prevented from foraging, frustration may arise, potentially increasing the risk of redirected behaviors, such as feather pecking (Schwarzer et al., 2022). The presence of the litter on the ground also triggers dustbathing and preening which reduces stress and promotes social bonds in laying hens (Vestergaard et al., 1997; Campbell et al., 2016).

As for the effect of microclimatic conditions, we observed that the rate of hens pecking on the ground increased from January to June at the expense of the rate of hens scratching and preening. In fact, in the previous paper of ours, Bordignon et al. (2025a) reported that hen presence on the ground progressively decreased from 27% to 19% of visible animals over the same period. We had hypothesized that differences in microclimatic conditions could have shaped the distribution of the hens among the different levels where in June air movement from the corresponding inlets into the barn likely provided more suitable conditions for heat dispersion at the upper level. On the other hand, differences in the number of animals (i.e. density) on the ground can affect the expression of ground pecking and scratching, and preening, which has to be confirmed, however.

Then, dustbathing is regarded as a synchronous behavior (Campbell et al., 2016) affected by several factors, such as genetic strain (space needs and tolerance for proximity), social influences (facilitation and dominance), time of day, space availability, and litter quantity and quality (Colson et al., 2007; Campbell et al., 2016; Grebey et al., 2020) besides environmental factors (i.e., temperature, radiant heat and light) (Duncan et al., 1998). As for the effect of the month, the changes we observed in the rate of dustbathing hens were numerically low (from a minimum of 4.91% in January to a maximum of 8.98% in March) and hardly related to the differences in microclimate conditions. In fact, differences in dustbathing have been previously recorded when comparing more extreme conditions (i.e., 22° C vs. 10° C) with increased number of dustbathing events (+50%) at higher temperatures (Duncan et al., 1998). On the other side, inadequate litter conditions significantly alter the expression of dustbathing in laying hens, resulting in incomplete or disrupted behavioral sequences (Moesta et al., 2008). Thus, we could expect that unsuitable control of environmental microclimate (as

for temperature and humidity) affect litter quality and, consequently, dust bathing activity, which however was not the case in our trial.

Finally, despite the occurrence of aggression being rather low (1.47% of observations on the ground), significant differences were found according to month. Some studies found a direct link between the number of hens on the ground and aggressive interactions (Nicol et al., 1999; Bilčík and Keeling et al., 2000), which was somewhat consistent with the higher rate of visible hens on the ground reported by Bordignon et al. (2025a) and the higher rate of aggression measured in the present trial in January. On the other hand, aggression is likely to decrease with the age of hens (31-33 vs. 37-40 weeks of age in January vs. March and June) as social hierarchies stabilize and activity levels decline (Hocking et al., 2001; Skomorucha and Sosnowska-Czajka, 2025).

As for piling, the microenvironmental conditions within the farming system, including temperature, relative humidity, lighting, and solar radiation, can increase the attractiveness of specific barn areas, promoting spatial clustering and aggregation of hens (Mazocco et al., 2024). Indeed, while under our conditions its occurrence was very low, piling is a problematic collective behavior in cage-free laying hens (Armstrong et al., 2023), which can escalate into smothering, i.e. the death of one or more birds. Nevertheless, the correlation between piling and microenvironmental conditions remains to be thoroughly investigated. On the other hand, age effects on piling behaviour (as for the frequency of occurrence and the duration) have been previously described in laying hens which have been related to increased space use in the aviary system with age (Winter et al., 2021).

As for behaviors on the tiers, we observed that the rate of perching hens increased from the first to the third tier consistently with the perch availability and the natural tendency of hens to select elevated resting sites, associated with reduced predation risk and increased perceived safety (Bist et al., 2023). As for the effect of the month, the decrease in the rate of perching hens we observed from January and March to June could be related to the differences in the spatial distribution of hens as reported by Bordignon et al. (2025a), i.e., the rate of hens at the upper tier increased from 24% and 26% to 34% in January and March to June, respectively ($P < 0.001$). As discussed above, this shift was likely shaped

by more favorable microclimate conditions for mitigation of heat stress in June at the upper tier, i.e., increased air movement and ventilation (Hu et al., 2021). On the other hand, increased hen density on the upper tier could have increased competition for perching space, increasing activity (i.e., tier pecking) and reducing comfort behaviors (i.e., preening) (Anderson et al., 2023). Indeed, other behavioural changes also accounts for the increased perching observed over time as locomotor activity decreases and static behaviours increase with age (Sokolowicz et al., 2020; Wang et al., 2021). As hens mature, their behavioural patterns also evolve driven by changes in their social dynamics and environmental preferences (Hüttner et al., 2023), where social reliance of young domestic and feral chickens has been found to weaken as they grow older (Wang et al., 2021). Accordingly, as hen age and month-/microclimatic conditions concurrently changed under our conditions, their individual effects on some specific behaviours cannot be completely separated.

4.2. Daily behavioral patterns across different microclimatic conditions

In laying hens, circadian rhythms regulate different processes, i.e., behavior, physiology, and productivity (Geng et al., 2022; Huang et al., 2026); they synchronize daily patterns of activity, including feeding, oviposition, and resting with the light-dark cycle (Geng et al., 2022). Laying hens forage mainly during the daytime, while resting and sleeping primarily during the dark period, with sleep serving a critical function in recovery from daily challenges and activities (Moss et al., 2023). Housing systems that interfere with nocturnal rest, such as those with light or disturbances during the dark phase, can compromise circadian organization, reduce recovery and compromise animal welfare (Yu and Li, 2023).

Environmental factors modulate circadian rhythms, with photoperiod, light intensity, and spectral composition primarily and strongly influencing behavioral and physiological synchronization (Geng et al., 2022). Then, heat stress has been shown to disrupt activity patterns and impair sleep behavior (Putyora et al., 2023). Within the farm, feeding schedules also interact with daily behavioral patterns, where the alignment of feed intake with the natural day-night cycle improves metabolic efficiency and reproductive performance (Toroghian et al., 2026).

Under our conditions, changes in the specific behaviors according to the observation hour (Fig. 1, Fig. 2) were consistent with previous results in aviary-housed hens (Campbell et al., 2016; Bessei et al., 2023; Yu and Li, 2023). Interactions between observation hour and month were not statistically significant (ground pecking) or, when significant (all other behaviors), showed erratic changes related to a few time windows (Fig. 3, Fig. 4). In detail, compared with the other months, the rates of preening hens (Fig. 3a) and ground scratching hens (Fig. 3b) were significantly higher in January at most observations hours; the rates of piling were higher between 9:00 and 15:00 in March (Fig. 3c). In other words, the daily behavioral pattern was consistent across different microclimatic conditions but showed month-specific differences at certain times. On average of the months, the higher rates of hens performing ground pecking and scratching in the late afternoon (Fig. 1), shortly before hens ascended to the upper levels for the night (Fig. 2) reflected an anticipatory foraging behavior, where hens search for feed before night roosting, as seen in wild fowl (Collias and Saichuae, 1967). In the wild, red junglefowls (*Gallus gallus*) also explore their surroundings in search of food before sunset to avoid foraging during periods of low visibility when predators are more active (Collias and Saichuae, 1967). Then, the midday hours (9:00 am to 16:00) during which the rate of dustbathing hens peaked (Fig. 1) were aligned with the time hens in our aviary had completed their morning feeding (3 feeding boots at 5:00, 8:00 and 12:00) and were engaged in comfort-related behaviors before the last feeding boot (16:00). This sequence reflects the fowl ethogram (Collias and Saichuae, 1967) and has been previously observed also in hens in other aviary systems (Campbell et al., 2016). In

fact, intense activities, such as foraging, usually occur in the morning and late afternoon hours, while preening and dustbathing during the central hours of the day (Campbell et al., 2016). Then, dustbathing typically takes place early in the afternoon (Campbell et al., 2016) for an approximate duration of 20-30 min per time (Van Liere et al., 1990). As for piling, it especially occurred from 14:00 to 17:00. Previous authors have found that piling events last longer during midday and afternoon compared to the morning or evening hours, although pile sizes are smaller in the afternoon compared with other periods (Winter et al., 2021).

Regarding behaviors on the upper levels of the aviary, perching followed a clear daily behavioral pattern: during nighttime hours, laying hens showed a consistent preference for upper tiers, which also had more perching sites in our aviary (Fig. 2). This behavior reflects the species' natural tendency to seek elevated roosting sites for protection during rest periods (Collias and Saichuae, 1967). While evolutionarily advantageous, in intensive systems such behavior can lead to overcrowding in upper tiers at night (Brendler and Schrader, 2016). Other factors affecting space use and animal distribution across the aviary, such as temperature, humidity and ventilation (David et al., 2015a; Bordignon et al., 2025a) could negatively affect natural perching behavior and increase negative interactions for perch competition (Anderson et al., 2023). Finally, out of our observations, preening also showed a consistent daily behavioral pattern, being expressed especially in the first hours of the day, as already described by other authors (Khalil et al., 2004), while detectable throughout the whole light time. Indeed, in pullets, preening has been described as one of the most synchronous behaviors, where spatial constraints (e.g. increased group size in the same space) reduce simultaneous preening (Keeling et al., 2017), but not preening frequency (Keeling, 1994).

5. Conclusion

Overall, under our conditions, laying hens exhibited a functional use of the multi-tier aviary space, expressing species-specific behaviors in areas equipped with appropriate resources and consistent daily behavioral patterns across different months and corresponding microclimatic conditions. Behavioral patterns on the ground and on the different tiers changed, which was likely related to a different distribution of the animals within the aviary depending on microclimatic conditions in the different months, despite month-specific differences at certain hours. Future research should consider more challenging and extreme microclimate conditions, both in indoor systems and, especially, in alternative outdoor systems to effectively address behavioral needs linked to climate change.

Ethical Approval for Animal Welfare

The experiment was approved by the Ethical Committee for Animal Experimentation (Organismo per la Protezione del Benessere Animale, OPBA) of the University of Padova (Project n. 28/2020; Prot. n. 204398; 06 May 2020). All procedures adhered to EU Directive 2010/63/EU (EU, 2010) guidelines for protecting animals used in scientific research.

CRediT authorship contribution statement

Mattia Pravato: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Francesco Bordignon:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Gerolamo Xiccato:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Giulia Zorzi:** Writing – review & editing, Investigation. **Andrea Pezzuolo:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Francesco Marinello:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Angela Trocino:** Writing – review &

editing, Writing – original draft, Visualization, Supervision, Resources, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be available on request from the corresponding author.

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