Animal 18 (2024) 101157

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Comb size, shape complexity and laterality of laying hens reared in environments varying in resource choice

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A R T I C L E I N F O

Article history:
Received 22 November 2023
Revised 29 March 2024
Accepted 4 April 2024
Available online 12 April 2024

Keywords:
Condition-dependent signalling
Environmental enrichment
Female ornamentation
Positive animal welfare
Stress resilience

A B S T R A C T

The comb is an ornament involved in signalling condition in domestic fowl. We hypothesised that comb size, comb shape complexity (i.e., rugosity, the comb perimeter jaggedness), and comb laterality of laying hens would be influenced by the degree of environmental enrichment experienced during juvenile development in the form of resource choice. We conducted a 2 × 2 factorial crossover experiment with pullets reared in pens containing four perches of equal length and four litter areas of equal size. Pullets were exposed to a single choice vs multiple choices of perch and litter types (i.e., all the same vs all different) during Weeks 1–4 (Period 1) and/or Weeks 5–15 (Period 2) of rearing (n = 4 pens/treatment combination) prior to transfer to standard adult laying pens for Weeks 16–27 (Period 3). In Week 27, combs were photographed, and comb laterality (hanging on left or right side) was noted. Using a custom-made image analysis programme, we captured comb area (mm^2), perimeter length (mm), and rugosity ((perimeter length − horizontal length) / horizontal length) from comb photographs of 6–7 randomly selected hens/pen. We predicted that hens reared in the multi-choice environment during Periods 1 and 2 would have larger, more complex, and left-side-biased combs than those in the other treatment groups, reflecting lower allostatic load. The predicted comb side bias was based on a possible bias in head posture/-movements associated with greater right eye/ear use and left-brain hemispheric dominance. Contrary to our predictions, we detected an overall right-side bias in comb laterality, and no associations between resource choice treatment in Period 1 or Period 2 and comb area, perimeter length, rugosity, or laterality of the adult hens. Thus, variation in allostatic load resulting from the rearing treatments was insufficient to modify the trajectory of comb morphological development, possibly due to a ceiling effect when comparing environmental treatments on the positive end of the welfare spectrum. We found that left-lopping combs had shorter perimeters than right-lopping combs. However, among hens with left-lopping combs, those with larger combs were heavier and had less feather damage, while among hens with right-lopping combs, those with longer-perimeter combs were heavier and tended to have less comb damage. In conclusion, comb characteristics were related to physical condition at the individual level but did not serve as sensitive integrated indicators of hen welfare in response to basic vs enhanced resource choice during rearing.

Implications

Laying hen combs function as condition-dependent signals of individual fitness, meaning comb quality should be enhanced under more beneficial environmental conditions. We expected hens raised in more heterogeneous environments, offering diverse perch and litter types, to have larger, more complex combs than counterparts raised in environments more like commercial production systems (one perch type; one litter type). However, although comb measures reflected individual physical condition, the rearing environment treatments had no effect on adult comb characteristics. Our results are relevant for poultry husbandry by indicating that comb characteristics signalled welfare differences between individuals but not good vs better rearing conditions.

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https://doi.org/10.1016/j.animal.2024.101157
1751-7311/

Introduction

Biological ornaments are conspicuous traits considered to function as condition-dependent signals of mate quality during mate selection (Hill, 2014; Winters, 2018), though they may serve a dual role as armaments (i.e., weapons or status badges; Berglund et al., 1996). Examples of visual ornamentation can be found across many taxa, such as the colourful iridescent tail feathers of peacocks (*Pavo cristatus*; Dakin and Montgomerie, 2013) and the long eye stalks of stalk-eyed flies (*Teleopsis dalmanni;* Cotton et al., 2010). As condition-dependent signals, ornaments are reported to have higher quality under more favourable environmental conditions (e.g., head ornaments of unparasitised vs parasitised male red jungle fowl, *Gallus gallus,* progenitor of the domestic fowl; Zuk et al., 1990). While male ornaments are generally more exaggerated, female ornaments can also provide information about their bearer's physical condition and reproductive potential (Nolazco et al., 2022), which is relevant for reciprocal mate selection and female resource competition (Kraaijeveld et al., 2007; Fitzpatrick and Servedio, 2018). For example, in choice tests, dominant feral domestic fowl males were found to mate sooner with females bearing relatively large head ornaments and to deposit more sperm when mating with them (Cornwallis and Birkhead, 2007).

Allostatic load can be defined as cumulative "wear and tear" affecting body condition and brain function that results from exposure to environmental challenges (McEwen, 1998). It derives from the continual adjustments made by bodily systems to maintain allostasis (i.e., optimal functioning in the face of changing demands; McEwen, 1998). While some allostatic load can be considered normal, high allostatic load can result from frequent stress, blunted negative feedback following stress, or unsuccessful stress responses (McEwen, 1998). Individuals vary in their accrual of allostatic load, even in response to similar challenges, due to differences in genetics and in how they perceive their environment (Korte et al., 2005). The degree of allostatic load may affect the elaboration of ornamentation, such that adult ornamentation serves as an integrated signal of life-to-date stress resilience and quality of life. Hence, larger, more complex ornaments may indicate better welfare earlier in life.

The comb is the fleshy head ornament of domestic fowl and red jungle fowl. While heritability estimates for domestic fowl comb size are high (0.61–0.69), the comb exhibits considerable phenotypic plasticity (Shen et al., 2016) consistent with functioning as a signal of individual fitness. In laying hens, comb features such as comb size and colour have been connected to social status (e.g., Siegel and Dudley, 1963; O'Connor et al., 2011) and to commercially important fitness-related measures such as BW (e.g., Tufvesson et al., 1999) and fecundity (Wright et al., 2012). The comb is rudimentary at hatch but develops during sexual maturation under the influence of androgen hormones (Mukhtar and Khan, 2012), with rapid growth beginning about 8 weeks before the onset of lay (i.e., during puberty) in laying pullets (Eitan et al., 1998). Consequently, comb growth will be impacted by any stress-related variation in the circulating androgen levels of females, especially during the period leading up to the onset of lay.

Comb types vary across strains of domestic fowl, with the "single" comb type being typical of strains reared for commercial egg production. As single combs grow, they reach a point where they start to tilt and eventually hang habitually (lop) to the right or left side of the head (Tufvesson et al., 1999; Wan et al., 2018). Mueller and Hutt (1942) reported that right-lopped combs were more common than left-lopped combs but that neither left-lopped fathers nor mothers consistently produced more left-lopped offspring. This finding suggests that comb laterality is influenced by environmental conditions during development. It is conceivable that comb laterality is related to individual differences in head posture and movements. The chicken brain shows lateralisation in the processing of sensory, social, and unfamiliar stimuli, with lateralisation in the use of the eyes, ears and nares when evaluating environmental stimuli (Rogers, 2023). In particular, there is a bias towards using the left eye when evaluating novel and potentially dangerous visual stimuli (Rogers, 2010). Thus, more anxious birds, that can be expected to accumulate higher allostatic load, may more frequently evaluate their environment using their left eye and ear than calmer chickens, potentially increasing the likelihood of developing a right-lopped comb.

Chicken comb shape varies in "jaggedness" of the outer combline (rugosity) between individuals, with differences in the number, width, and height of the points. In adults, comb shape appears to remain relatively stable over time, consistent with a role in individual recognition (Guhl and Ortman, 1953). Comb shape has been associated with specific genes (Bakovic et al., 2022) but can also be altered by environmental factors such as frostbite, accidental tearing and pecking injuries. Thus, environmental conditions during rearing may play a role in sculpting adult comb shape complexity as well as influencing comb size.

In commercial aviary rearing systems, chicks are often kept in cage-like compartments for their first 4–8 weeks, after which the compartments are opened and the whole aviary including the litter floor becomes accessible. At around 16 weeks of age, the pullets are moved to an aviary house optimised for egg production, where they begin to lay eggs at around 18–22 weeks of age and remain throughout adulthood. Exposure to environmental change presents challenges (e.g., Brantsæter et al., 2016) that may contribute to allostatic load. Providing a more complex and heterogeneous environment during juvenile development, both prior to the opening of rearing compartments and prior to the move to adult housing, may improve the birds' ability to adapt to environmental changes, minimising allostatic load and enhancing their ability to grow an elaborate comb. In support of this hypothesis, Nazar et al. (2022) and Skånberg et al. (2023) found that laying hen chicks reared for 3–4 weeks with access to multiple variants of perching structures and litter materials were less fearful and had greater adaptability when exposed to environmental change compared to chicks that were kept with only a single variant of each resource type.

A subsequent longer-term cross-over experiment adds support for the idea that laying hen comb morphology could be affected by resource choice during ontogeny. We investigated the effects of resource choice during the chick (Period 1) and pullet (Period 2) rearing stages on outcomes in early adulthood (Period 3) when all birds were kept in the same environment (Holt et al., 2024a). Hens were reared with one perch type and one litter type (single-choice, representing a basic level of environmental enrichment), or four variants of perches and litter (multi-choice, representing an enhanced level of environmental enrichment), either throughout both Periods 1 and 2 or in succession with order counterbalanced across groups. Treatment differences in behaviour, growth, and plumage condition suggested that allostatic load across Periods 1–3 was lower in multi-choice than single-choice hens. To explore the potential effect of resource choice during rearing on comb development, the current investigation utilised comb data collected from the adult hens at the end of Period 3, coinciding with peak daily egg production.

We hypothesised that comb development would be affected by the amount of resource choice available to pullets during rearing. Specifically, we predicted that hens kept in the multi-choice environment during Periods 1 and 2 would have larger, more complex, and more left-side-biased combs at the end of Period 3, reflecting lower allostatic load, compared to hens kept in the single-choice environment in both periods. The design of our experiment also allowed us to investigate whether the timing and order of exposure to a multi-choice environment (Period 1 or Period 2) would differentially influence comb development. We expected that exposure to the multi-choice environment in either period would result in more elaborated combs than single-choice throughout rearing. Nonetheless, we predicted that the effect of the multi-choice environment would be larger in Period 2 than in Period 1 due to rapid comb growth associated with sexual maturation in that period. In addition to treatment effects, we also investigated variation in comb traits in relation to individual physical condition. We expected to find positive correlations between comb size metrics, comb shape complexity and BW of individual hens, and negative correlations of these variables with feather and comb damage scores. Furthermore, we predicted that hens with left-lopped combs ("**lefties**") would be heavier, with larger, more complex combs and lower feather and comb damage scores, than hens with right-lopped combs ("**righties**").

Material and methods

Animals, housing, and management

We conducted the study at the Swedish Livestock Research Centre of the Swedish University of Agricultural Sciences in Uppsala, SE. We obtained day-old laying hen chicks (Bovans Robust chicks, n = 364) with intact beaks from a local hatchery and assigned them to 16 visually isolated rearing pens (240 × 120 × 180 cm) in groups of 22–23 birds (balanced for group weight). Each pen contained a drinker line with four water nipples, two circular chick feeders, four 120-cm-long perches, and four shallow trays containing litter (71 × 35 × 3.5 cm) that were emptied and refilled at least once weekly as needed to maintain the quality and quantity of their contents. In Week 3, we replaced the chick feeders with a round hanging feed hopper. We increased the perch height from 15 to 45 cm in Week 3 and to 55 cm in Week 5, when we exchanged the shallow litter trays for deeper trays (78 × 56 × 18 cm). We gave *ad libitum* access to water and standard starter feed for the first 6 weeks, followed by standard growing feed. The room temperature was set at 25 °C for the first 9 weeks and then dropped to 20 °C. A hanging heat lamp provided additional warmth at chick level for the first 4 weeks. The photoperiod was 20 h on Day 1 and gradually reduced to 10 h by Week 6. Mean (± SE) light intensity (measured in the front and back half of each pen) was 18 ± 1.32 lx at bird level, except for a 15-min dawn and dusk period at the beginning and end of each photoperiod.

In Week 16, we transferred the birds in their groups to 16 adult laying pens (362 × 356 × 297 cm). These pens had a 132 × 356 cm solid-floored littered area and a raised slatted area (230 × 356 cm), as well as two colony nests, two hanging feed hoppers, a bell drinker, five elevated perches, three elevated platform nests and five low perches attached to the slats. Room temperature was set at 20 °C and light intensity at hen level (measured in the litter and slat areas) averaged 5.4 ± 0.21 lux. The photoperiod was 10 h in Weeks 16–19, then increased by 1 h weekly to 14 h. At the end of the experiment (Week 27), we adopted the hens out to local poultry keepers.

Experimental design

The study was divided into three experimental periods. In Period 1 (Weeks 1–4, chick–rearing period), we assigned chicks to one of two treatments (Fig. 1a) in a randomised block design: Single-choice or Multi-choice. Single-choice pens (n = 8) had four perches of the same type (a round rubber perch, a braided cotton rope, a wire mesh perch, or a wooden plank) and one litter type in all four litter trays (straw, wood shavings, peat moss, or fine sand). We formed four unique perch-litter combinations that were balanced across the Single-choice pens (Fig. 1b). Each Multi-choice pen (n = 8) had all four different perch types and all four different litter types (one litter type/tray). We balanced the location of each resource type within the pen across the Multi-choice pens. In Period 2 (Weeks 5–15, pullet rearing period), we switched the treatment in half the pens (n = 4 pens/treatment) to the opposite treatment (Fig. 1a). In Period 3 (Weeks 16–27, adult laying period), all groups were housed in standard laying pens with resource types not experienced during rearing. These included crushed straw pellets as litter and a variety of wooden and plastic perches of novel dimensions. See Holt et al. (2024a) for further details regarding the experimental conditions.

Data collection

We collected data at the end of Period 3 (Days 184–186). Each hen was weighed and scored for feather and comb damage (Table 1), and the side of the head to which her comb lopped (right or left, i.e., comb laterality) was noted (Fig. 2a, b). One researcher then laid the bird on her side on a table with her comb (inner side facing up) lying on a black clipboard with an attached ruler while another photographed the comb from directly above, with the ruler in view to provide the scale (Fig. 2c, d). After excluding 10 photographs with blurry or buckled comb images, we extracted comb measurements (Table 1) from the photographs of 6–7 randomly selected birds per pen (100 birds in total, based on power analysis to calculate the number of comb pictures required to reach power > 0.80).

We determined the maximum length and height of each comb, as well as the area and perimeter length of the comb, employing a custom-made image analysis programme written in C++ programming language for Linux (Ubuntu 20.04; Canonical Ltd., 2020). The first step was to calculate the image pixel size (width and height in mm) based on the number of pixels along a 10-mm length of the ruler in the image. As the comb was uneven and head movements could occur, there was unpredictable variation in light intensity across the comb. Therefore, to segment the comb, colour values were selected from a drop-down menu and image thresholding was applied to build up a binary (black and white) image of the comb. Once the comb was marked, additional filtering (closing, dilation, eroding) was used to refine the image. With the aid of the OpenCV blob detector (Mallick, 2024) and Canny edge detector (OpenCV, 2024), all required comb measurements were then extracted. With two clicks on the image, the programme also enabled manual measurement of any dimension of interest. The output was exported to a spreadsheet for statistical analysis. As a measure of comb shape complexity controlling for comb size, we calculated a rugosity index by subtracting the comb length from the perimeter length and dividing the difference by the comb length (Table 1). This measure equates to the classical assessment of rugosity by draping a flexible transect line over an uneven surface and calculating its length relative to the flat distance between the two endpoints.

Quality assurance

All comb data were collected by one observer. To assess inter-observer concordance, a second observer used the same custom-made image analysis programme to extract comb area and comb perimeter data from 16 birds (one randomly selected bird/pen; four birds/treatment). The results indicated good agreement between observers (mean intraclass correlation = 0.88, calculated using the package psych; Revelle, 2023). To validate our custom-made image analysis programme, we compared the comb area

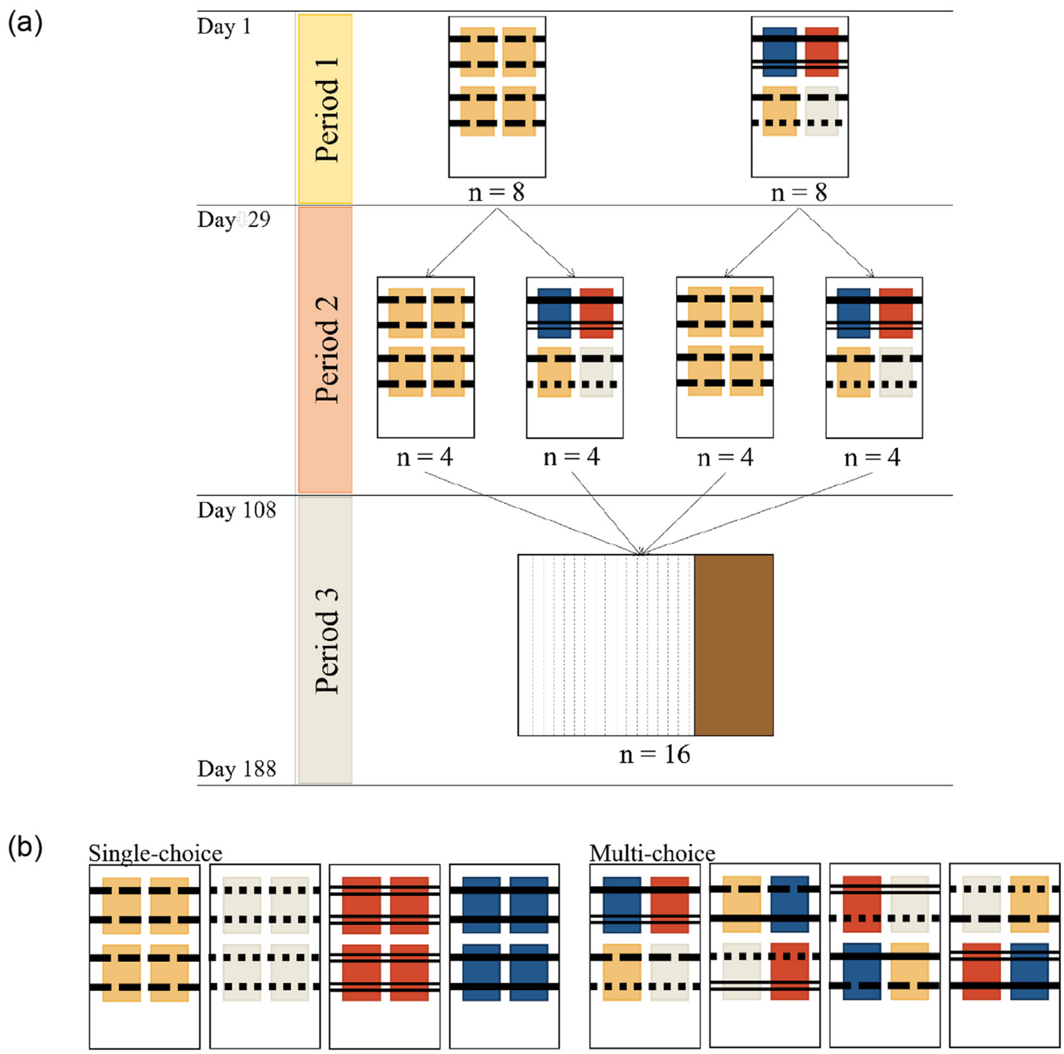

Fig. 1. Experimental design. (a) Experimental timeline, illustrated with one exemplar of each treatment. In Period 1 (Weeks 1–4), groups (n = 16) of laying hen chicks were assigned to one of two treatments: Single-choice, with one of four possible perch types (black lines) and litter types (coloured rectangles), or Multi-choice, with all four perch and litter types. In Period 2 (Weeks 5–15), half the groups were switched to the opposite treatment. All groups were moved to similar pens for Period 3 (Weeks 16–27) that consisted of a slatted floor (white rectangle) with several novel perch types and a large litter area with one novel litter type, crushed straw pellets (brown rectangle). (b) The four combinations of perch and litter types used in the Single-choice treatment (balanced across replicate pens): rubber rod (dashed line) + straw (yellow), rope (dotted line) + wood shavings (pale beige), wire (double line) + peat (red), wooden plank (solid line) + sand (blue), and the four combinations of perch and litter locations used in the Multi-choice treatment (balanced across pen locations in replicate pens).

and perimeter results from the 16 birds with results from the same birds obtained using the established programme, ImageJ (Schindelin et al., 2012). The mean intraclass correlation was 0.93, indicating high reliability.

Statistical analysis

Statistical analyses were conducted in RStudio (RStudio Team, 2023) using R 4.3.1 (R Core Team, 2023), with alpha = 0.05. We employed residual diagnostics plots produced by the DHARMa package (Hartig, 2022) to confirm model fit and conformance with assumptions, and the emmeans package (Lenth et al., 2023) to obtain estimated marginal means and confidence intervals. To examine treatment effects, we evaluated the impact of Period 1 treatment, Period 2 treatment and their interaction on the comb variables: comb area, comb perimeter length, comb shape complexity and comb laterality. (For treatment effects on BW, and feather and comb damage scores, see Holt et al., 2024a). Comb length and height were closely related to comb area and perimeter and were excluded from the analysis to avoid redundancy, retaining comb area and perimeter as analysed measures of comb size. The continuous variables (comb area, comb perimeter length, comb shape complexity) were analysed using linear mixed models (lme4 package; Bates et al., 2015) with Gaussian distribution, fitted with restricted maximum likelihood. The Satterthwaite df approximation was applied to *t*-tests for these models. We examined comb laterality, a nominal variable, in a generalised linear mixed model (lmerTest package; Kuznetsova et al., 2017) with binomial distribution, maximum likelihood parameter estimation and Laplace approximation. A Chi-square test was employed to assess whether combs lopped more to one side than expected by chance.

We used linear mixed models to compare comb measurements, BW and feather damage scores of hens with right- vs left-lopped combs, while their comb damage scores were compared by means of a cumulative link mixed model with maximum likelihood parameter estimation and Laplace approximation (ordinal package; Christensen, 2022; RVAideMemoire package; Hervé, 2023). Due to collection of data from multiple hens per pen, pen was

Table 1
Comb and physical condition measures collected from adult laying hens.

Variable	Description
Comb traits	
Comb length (mm)	The longest horizontal length of the comb parallel to a line from the front of the head to the back of head
Comb height (mm)	The greatest vertical height of the comb from the top of the head to the tip of the highest comb peak, perpendicular to the comb length.
Comb area (mm^2)	The two-dimensional surface area of the inner side of the comb (side adjacent to the head) as a measure of comb size.
Comb perimeter length (mm)	Continuous line tracing the complete boundary of the comb, as a measure of comb size influenced by comb shape.
Comb shape complexity	Index of rugosity calculated as (comb perimeter – comb length) / comb length, representing comb shape controlling for size.
Comb laterality (n of birds)	Lop of the comb to the left or right side of the head.
Physical condition measures	
BW (g)	Individual hen weight to nearest g.
Feather damage score (0–4)[1]	Proportion of scruffy, split, broken or missing feathers, visually scored as 0 (0%), 1 (1–24%), 2 (25–49%), 3 (50–74%) or 4 (75–100%) on each of six body regions (head, neck, wing coverts/primaries, back/rump, belly, tail), averaged to obtain a mean score.
Comb damage score (1–3)	Number of peck wounds on the comb, scored as 1 (0–3), 2 (4–6) or 3 (>6).

[1] Based on the scoring system of Bilcik and Keeling (1999) but, because birds in this study showed less severe feather damage and loss, we modified the scoring system in order to detect variation in feather condition.

the random effect in all mixed models. Due to the randomised block design, experimental block was included in initial models but had no effects and was excluded from the final models. To evaluate associations between the variables, Pearson correlations between the comb traits, BW and feather and comb damage scores were calculated separately for "righties" and "lefties".

Results

Comb response to resource choice

None of the analysed comb variables were significantly affected by the treatments experienced during Period 1, Period 2, or their interaction (Table 2). Descriptive statistics on the measured variables are presented in Table 3.

Comb laterality

Of the 100 birds sampled, 76 hens were "righties" while the remaining 24 were "lefties". This right-side bias was greater than that expected in the absence of laterality (i.e., with a 50% chance of the comb lopping in either direction; χ^2 = 13.52, df = 1, $P < 0.001$). "Righties" had combs with longer perimeters than "lefties" ($t_{98.0}$ = 2.24; P = 0.028) and tended to have combs with greater shape complexity ($t_{96.9}$ = 1.94; P = 0.055; Fig. 3). Results for the remaining analysed comb variables did not differ between "righties" and "lefties" (see Supplementary Table S1 for estimates).

Comb trait correlations

Comb perimeter was positively correlated with comb area ("righties" and "lefties": $P < 0.001$) and comb shape complexity ("righties": $P < 0.001$; "lefties": P = 0.010; Fig. 4). BW was weakly positively correlated with both comb area ("righties": P = 0.056; "lefties": P = 0.021) and comb perimeter length ("righties": P = 0.022; "lefties": P = 0.085). In "lefties", heavier hens (P = 0.036), hens with bigger comb areas (P = 0.013), and hens with longer comb perimeters (P = 0.042) sustained less feather damage whereas among "righties", hens with bigger comb areas (P = 0.082) and hens with longer perimeters (P = 0.052) tended to have less comb damage.

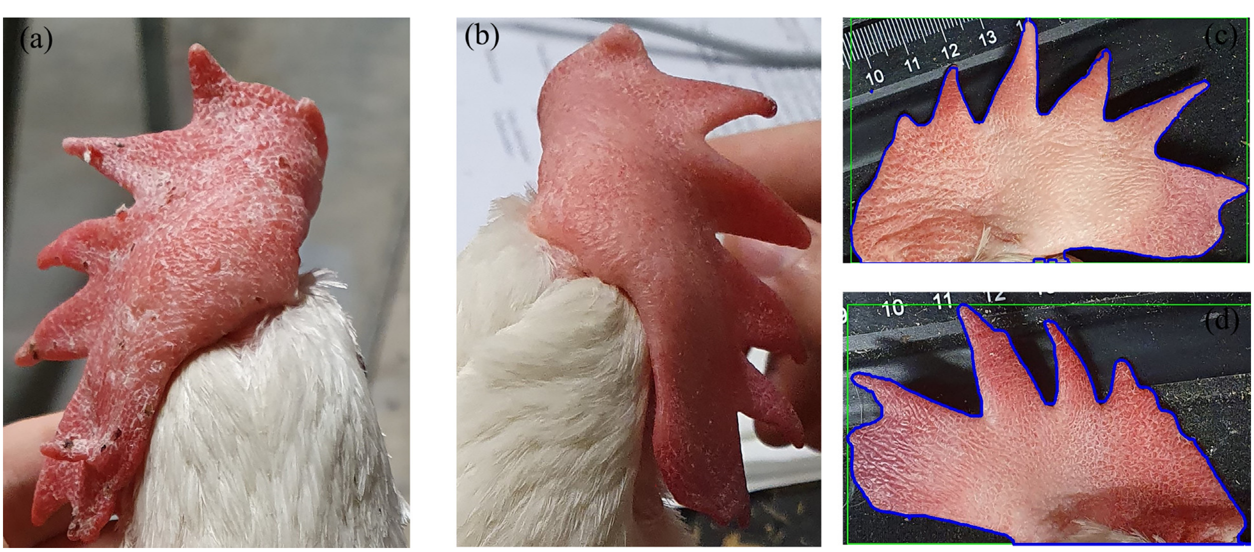

Fig. 2. Laying hen combs at 184–186 days of age: (a) a hen with a left-lopped comb; (b) a hen with a right-lopped comb; (c–d) examples of outputs from a custom-made image analysis programme showing measurement of comb length and height (green box), and comb area and perimeter length (blue outline), of the inner surface of a left-lopped comb (c) and a right-lopped comb (d) based on the picture scale (n pixels/10 mm). See Supplementary Figure S1 for uncropped photographs with further details.

Table 2
Effects of rearing treatments (single- vs multiple choices of perch and litter types) experienced in Period 1 (Weeks 1 – 4), Period 2 (Weeks 5 – 15) and their interaction on comb condition of 184–186-day-old laying hens (n = 100). Estimates with 95% confidence interval (CI) come from linear mixed models (*t* statistic) and, for comb laterality, from a generalised linear mixed model (*Z*-statistic). The single-choice treatment was the reference treatment.

Response variable	Predictor	Estimate	95% CI	*t*- or *Z*-value	df	*P*-value
Comb area	Period 1	139.5	−88.67 – 396.60	1.10	96	0.276
	Period 2	85.6	−163.17 – 352.08	0.67	96	0.503
	Period 1 × Period 2	−220.1	−555.74 – 120.51	−1.22	96	0.224
Comb perimeter	Period 1	−4.2	−29.33 – 20.31	−0.33	96	0.746
	Period 2	4.2	−19.75 – 30.25	0.32	96	0.746
	Period 1 × Period 2	2.4	−34.73 – 37.86	0.13	96	0.894
Comb shape complexity	Period 1	−0.1	−0.31 – 0.15	−0.63	12.5	0.539
	Period 2	0.1	−0.18 – 0.29	0.62	12.5	0.545
	Period 1 × Period 2	0	−0.33 – 0.35	−0.03	12.5	0.979
Comb laterality	Period 1	−0.3	−0.29 – 0.42	−0.33	Infinity	0.739
	Period 2	0.2	−0.15 – 0.31	0.22	Infinity	0.825
	Period 1 × Period 2	1.0	−0.34 – 0.33	0.79	Infinity	0.431

Table 3
Comb and physical condition measurements (overall mean ± SE) of laying hens (n = 100) at 184–186 days of age (end of Period 3).

Variable	Mean	SE
Comb traits		
Comb length (mm)	85.2	0.81
Comb height (mm)	47.6	0.65
Comb area (mm^2)	2 359.0	44.70
Comb perimeter length (mm)	320.0	4.50
Comb shape complexity	2.8	0.04
Comb laterality (n of birds)	24 left	76 right
Physical condition measures		
BW (g)	1 579.7	10.87
Feather damage score (0–4)	1.3	0.05
Comb damage score (1–3)	2.2	0.07

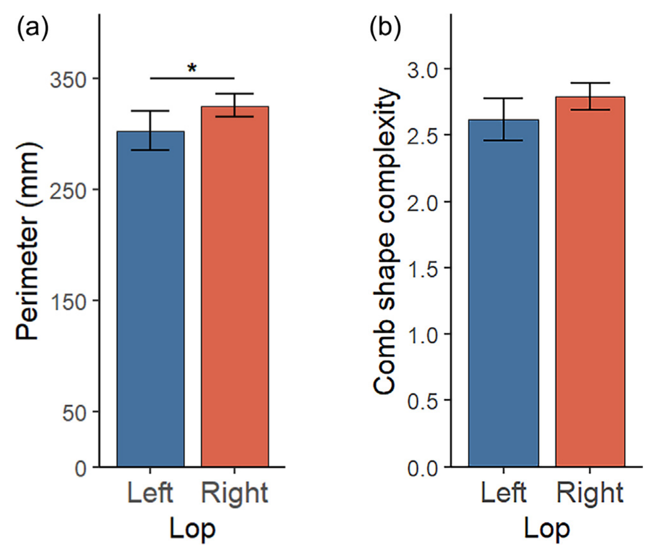

Fig. 3. Differences between hens with left- and right-lopping combs (mean with 95% confidence interval; * indicates difference at $P < 0.05$) in a) Comb perimeter length (mm; $P = 0.028$), and b) Comb shape complexity ($P = 0.055$).

Discussion

Stressors experienced during development, such as those related to exposure to a novel environment or pecks from flock mates, may increase allostatic load via the hypothalamic–pituitary–adrenal axis and inhibit androgen production, thus curbing the development of condition-dependent signals such as the comb of laying hens. Enriching the environment with resource choices during rearing, thereby increasing opportunities for learning and satisfaction of behavioural preferences, may buffer against the build-up of allostatic load by creating more contented, adaptable, and stress-resilient hens (Nazar et al., 2022; Skånberg et al., 2023; Holt et al., 2024a). On this basis, we predicted that hens reared in the more complex Multi-choice environment would develop larger, more complex, and left-side-biased combs compared to those reared in the less complex Single-choice environment. However, we did not detect significant differences in any of the comb measures between hens kept in the Multi-choice vs the Single-choice environment. There was neither an additive effect of the duration of exposure to the Multi-choice vs Single-choice environment nor an interactive effect related to the order of exposure to these environments. While we did detect some beneficial effects of the Multi-choice treatment on behaviour and body condition (Holt et al., 2024a), it appears that any treatment-related differences in allostatic load were too small to produce consistent, long-lasting differences in comb traits.

We aimed to compare a good with an even better environment and apply measures of comb condition as fitness-related cumulative indicators of positive welfare. This is in contrast to enrichment studies comparing a "barren" environment with an environment enriched to some degree. Although assessment of animal welfare is generally relative, making it unclear if enrichment-induced improvements indicate good welfare or "less poor" welfare, both environments in our study provided plentiful resources including ample access to perches and frequently refreshed litter. Our results suggest that our treatment comparison was operating on the positive end of the welfare spectrum, where a ceiling effect may have limited rearing treatment differences in the morphological development of laying hen combs. It is possible that treatment-related comb differences might have become apparent if the hens had been kept to an older age. However, we expected differences indicating good welfare would be most apparent during the juvenile and young adult period of comb growth.

Contrary to our prediction of a more left-sided comb bias in hens reared with resource choice, we observed a significant overall right-side comb bias in our hens. We found only one previous investigation of comb laterality in adult domestic fowl. Consistent with our finding of a 76% population bias in favour of "righties", Mueller and Hutt (1942) observed that 72.9% of 6 625 hens from four different strains had right-lopping combs. While the direction of lopping was stable in adults, they found no clear genetic explanation for the right-side bias, though it has subsequently been demonstrated that frequency-dependent population-level laterality could arise as an evolutionarily stable strategy (Ghirlanda et al., 2009). At the proximate level, the sidedness of combs may have been influenced by incubation in the dark, which is typical in commercial hatchery practice. Chicks incubated in the dark are reported to exhibit greater fearfulness than those exposed to light during incubation (Archer and Mench, 2017; Ruiz-Raya and Velando, 2022; Manet et al., 2023). Shy birds could be expected to frequently monitor the environment for danger, which is typically performed using the left eye and ear (Rogers, 2010). The resulting habitual head tilts could have contributed to a preponderance of "righties". Because "lefties" were less common, they may have been harassed by other hens (Dennis et al., 2008), which might explain their shorter comb perimeters and tendency for lower comb shape complexity in our study. Yet the lack of other systematic differences between the "righties" and "lefties" argues against this explanation. Moreover, Mueller and Hutt (1942) observed no difference in mortality, age or BW at first egg, egg production, or chick viability of "righties" vs "lefties". On a practical note, after combs have lopped to one side or the other, their continued growth can lead to obstruction of vision on that side, which may have implications for how hens perceive their surroundings. Large pendulous combs may impair net welfare unless their excessive size provides compensatory benefits such as improved thermoregulation in hot climates.

At the individual level, we detected a weak correlation between BW and both comb area and comb perimeter length of hens in Week 27, when egg production was peaking. This finding is consistent with other studies reporting a correlation between BW and comb size measures in laying hens (e.g., Tufvesson et al., 1999). However, not all studies have detected a correlation (e.g., Wright et al., 2012). Wan et al. (2018) found a correlation at 24 weeks of age, but not before or after this age, when comparing different breeds of laying hens between the ages of 4–30 weeks. These findings suggest that BW and comb size may be more tightly linked when comb growth is maximal (around puberty), especially in males given that they grow faster and larger than females. It can also be expected that the correlations between BW, comb area and comb perimeter length would manifest more strongly if welfare was compromised by prolonged undernutrition, such as if food intake was limited due to competition, disease, or natural incubation of eggs, or if food reserves were drained by adverse weather conditions or parasitism (e.g., Zuk et al., 1990). This was not the case in our healthy population of young hens with *ad libitum* access to food and other resources, where the hens' opportunity to reach their genetic potential for egg production was high. We also note that relationships between comb traits and reproductive outcomes can vary between selection lines (McGary et al., 2002).

We explored associations between plumage condition and comb measures as Holt et al. (2024a) found that, at the group level, birds exposed to the Multi-choice environment had less feather damage than birds exposed to the Single-choice environment, which was accompanied by a lower level of severe feather pecking behaviour in Periods 1 and 2, and a higher rate of dustbathing in Periods 2 and 3. We found a negative relationship between the mean feather damage scores of individuals and their comb area and perimeter length, although this was significant only in the less common "lefties". Overall, the differences in feather damage were relatively minor, being mainly due to differences in feather scruffiness, with limited feather splitting or breakage and no observations of heavy feather loss from any of the six evaluated body regions. Our results for "righties" are consistent with Tahamtani et al.'s (2017) finding of no difference in laying hen comb size between feather peckers, their victims, or control hens that were in neither of these categories. Although we did not find an association between the resource choice treatments and comb damage (Holt et al., 2024a), among the "righties", those with bigger comb areas and longer comb perimeters tended to have less comb damage. This finding is in keeping with evidence that laying hens with larger combs are more likely to win agonistic encounters and to

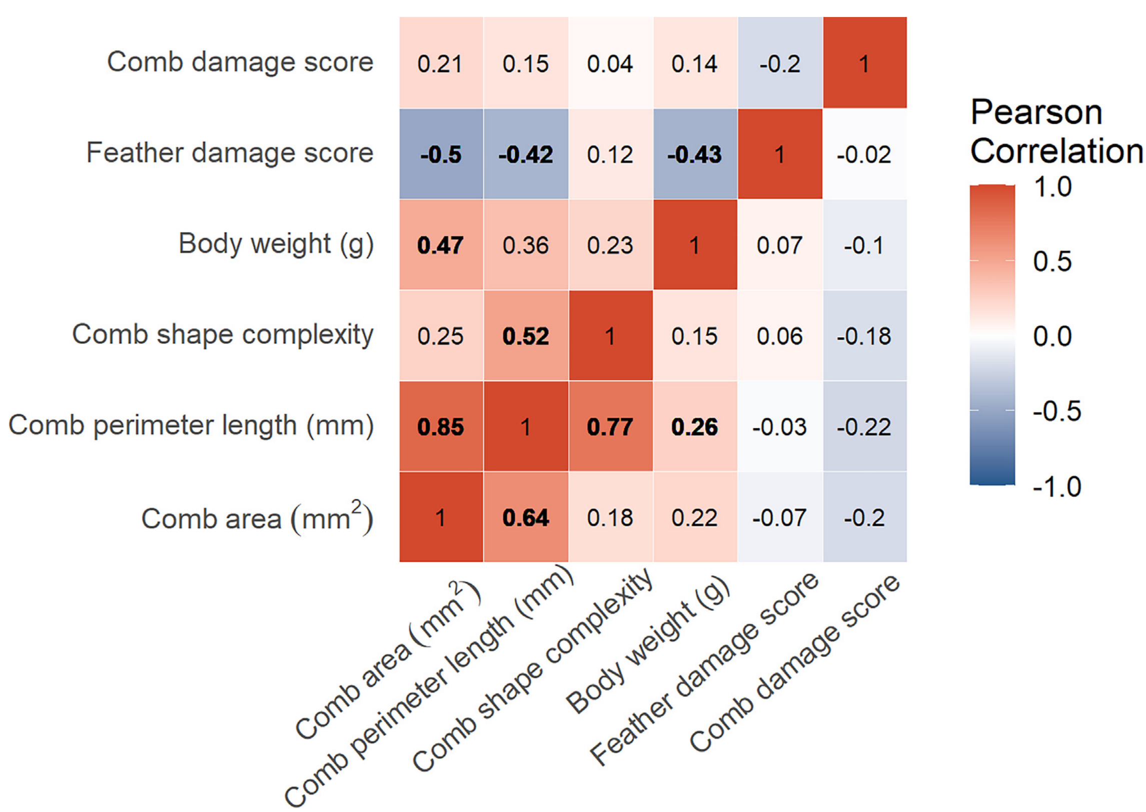

Fig. 4. Heatmap of correlations (*r* values) between comb and physical condition variables of laying hens at 184–186 days age; with values for hens with left-lopping combs (n = 24) above the diagonal and right-lopping combs (n = 76) below the diagonal. Bold text indicates correlations with $P < 0.05$.

maintain a higher social status than hens with smaller combs (e.g., Marks et al., 1960; Siegel and Dudley, 1963; Cloutier and Newberry, 2000; O'Connor et al., 2011).

While we detected some correlations between morphological comb traits and indicators of physical condition, other more rapidly varying comb characteristics may be better suited as current welfare indicators. For example, Ross et al. (2020) used the decrease in comb temperature and latency to return to basal comb temperature as measures of stress resilience. Comb colour can be another useful short-term welfare indicator (e.g., Zuk et al., 1990) if reliably measured without disturbing the birds.

Conclusion

Exposure to the Multi-choice condition during rearing served as an effective form of environmental enrichment with a long-lasting beneficial impact on the group-level welfare of hens as indicated by improved adult plumage condition, higher BWs and lower aggression (Holt et al., 2024a). However, the comb characteristics of the adult hens were unaffected by the rearing treatments, arguing against their use as reliable integrative indicators of the impact of rearing conditions on adult hen welfare, at least when comparing results from rearing environments with a basic vs enhanced degree of enrichment (i.e., good vs better environments) when a ceiling effect may apply. Because combs continue to grow after pullets are moved to adult housing, this finding does not discount the possibility that the measured comb characteristics can differentiate between adult environmental treatments. Factors affecting comb laterality, and the relevance of left- vs right-side bias for hen welfare, require further investigation. The correlations between adult comb and physical condition measures suggest that comb characteristics are useful animal-based indicators of individual differences in hen welfare, an important consideration even in the best of physical environments.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2024.101157.

Ethics approval

This study was performed on a common production species, typically kept indoors under human care. We kept the birds in small, stable groups at low density throughout the experiment (Periods 1 and 2: minimum 1 252 cm^2/bird, Period 3: minimum 5 603 cm^2/bird). All birds were familiar with the close proximity of, and handling by, the involved personnel. All procedures were approved by the Uppsala Animal Experiment Ethics Board (Number 5.8.18-11549/2017).

Data and model availability statement

The data and R code used to produce the statistics and figures presented in this article are publicly available on Github: https://github.com/RVHolt/ChoiceCombs.git (Holt et al., 2024b). Access to the image analysis source code may be given upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

Acknowledgements

The authors wish to thank the research facility staff and university students that helped with the daily care of the animals and data collection. An earlier version of the manuscript is included in the PhD thesis of Regine Victoria Holt.

Financial support statement

RVH was supported by a Ph.D. scholarship from the Norwegian University of Life Sciences (financed by the Norwegian Ministry of Education and Research) and an Erasmus + travel grant (Erasmus code: N AS03). The experiment and salary of LS were funded by a grant from FORMAS (Swedish Research Council for Sustainable Development, grant number: 2016-01761) to LJK, RCN and IE.

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