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The influence of genetic strain on fear and anxiety responses of laying hens housed in a cage-free environment

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ABSTRACT

Cage-free environments provide more behavioral opportunities for hens than cages, but fear responses in such open housing can lead to injuries and challenging human-animal interactions. This study evaluated the impact of genetic strain on fear and anxiety responses in two brown and one white genetic strain of laying hens: Hy-Line Brown (HB), Bovan Brown (BB), and H&N White (HN). Hens were assessed at the start of lay and peak lay through the inversion and attention bias tests, along with thermal imaging and core body temperature measurements to assess stress-induced hyperthermia. During the inversion test, HB hens performed significantly more wing flaps than other strains ($p=0.012$), while BB hens exhibited more vocalizations than HN hens ($p=0.0041$). Thermal imaging revealed that at the start of lay, HB and HN hens had higher maximum comb temperatures than BB hens ($p<0.0001$), but HB hens had lower temperatures at peak lay ($p=0.027$). BB and HN hens had higher core body temperatures at 4- and 5-minutes post-inversion ($p<0.0001$). In the attention bias test, HB and BB hens were more likely to resume eating and showed increased head bobbing as they aged, whereas HN hens were less likely to resume eating but maintained high head bobbing ($p=0.017$; $p=0.00056$). BB hens had the lowest average eye and maximum comb temperatures 3.5 to 4.5 min post-startle at the start of lay ($p<0.05$), white HN hens had the highest average eye and comb temperatures ($p<0.0001$) and higher average eye temperatures than BB at peak lay ($p=0.026$). Finally, HN hens had higher core body temperatures than HB hens at both the start of lay and peak lay ($p=0.041$; $p=0.046$). These results indicate that brown and white strains differ in their responses to fear and anxiety, with brown strains being more behaviorally responsive and white strains showing greater physiological stress. These strain-specific coping mechanisms provide insight into how hens may react to stressors in cage-free environments, aiding in strain selection for producers.

Introduction

As cage-free egg production increases in the United States, it is imperative to identify management practices that promote animal welfare, such as selecting genetic strains that will thrive in cage-free housing. Compared to conventional cages, cage-free laying hens have more space per bird, larger social groups, less confinement, and consequently more opportunities to express highly motivated behaviors (Hartcher and Jones, 2017). Simultaneously, cage-free laying hens may experience and react aversively to unexpected stimuli in complex cage-free housing, such as sudden noises, changes in light, and caretaker movements. Fear behavior is a short-term response to an immediate threat (Campbell et al., 2022). Common fear responses in laying hens include the “fight or flight” or freeze reactions (Anderson et al., 2021). These responses align with different coping styles, where proactive hens display active avoidance behaviors, such as fleeing, while reactive hens exhibit passive avoidance behaviors, such as freezing (Pusch et al., 2018). Flighty hens are susceptible to injury in cage-free housing if their fear behavior manifests in piling and smothering (Winter et al., 2021) or collisions with structures in the house (Harlander-Matauschek et al., 2015; Toscano et al., 2020). Genetic strains differ in their fearfulness temperament (Nelson et al., 2020; Rentsch et al., 2023), which is an important consideration for their suitability for cage-free housing.

To assess fearfulness in laying hens, the tonic immobility (TI) and the novel object tests (NOT) have been predominantly used in previous research. The TI test assesses the duration of time animals remain in a catatonic state to evaluate the freeze fear response (Anderson et al., 2021). The longer an animal remains in this state, the more fearful it is

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(Forkman et al., 2007). The NOT measures fearfulness responses to an unfamiliar item by quantifying the latency to approach and touch the object, where longer latencies indicate higher levels of fearfulness (Forkman et al., 2007). The context of the two tests differs, resulting in inconsistent conclusions about fearfulness in genetic strains. Previous research deemed white strains as more fearful during TI, while brown strains were considered more fearful with the NOT (reviewed by Rentsch et al., 2023). Both fearfulness tests also include confounding factors that complicates interpreting fearfulness responses in different genetic strains. For instance, Rentsch et al. (2023) reported that the time limit commonly placed on the TI test is not long enough to accurately measure the catatonic duration in white strains. Studies implementing the NOT differed in their methodology (e.g., individual versus group testing, type of novel object used), which makes it difficult to compare the behavioral responses of hens between studies (Rentsch et al., 2023).

The inversion test and attention bias test are additional assessments of fearfulness and anxiety that have better construct validity for behavioral responses in cage-free housing. Both tests involve sudden events that are likely to be experienced in cage-free environments, such as animal handling and unexpected noises. The inversion test is conducted by inverting a bird and restraining both legs with one hand (Archer and Mench, 2014), where the intensity of wing flapping (number of flaps/sec) quantifies the “flight” response (Newberry and Blair, 1993). In cage-free housing, flightiness could result in a potential injury if a hen collided with a structure (Stratman et al., 2015). Nelson et al. (2020) discovered that White Leghorn hens displayed a lower wing flapping intensity score and performed less wing flaps overall compared to Rhode Island Red hens. Similarly, Brown et al. (2022) found that Hy-Line Brown hens exhibited a higher wing flapping intensity compared to hens from three White Leghorn strains. These patterns demonstrate distinct strategies that brown and white strains utilize in response to the same handling stimulus (Nelson et al., 2020).

The attention bias test offers a means to assess anxiety, a long-term response to a potential threat (Lourenço da Silva et al., 2024), using a sudden auditory stimulus such as a conspecific alarm call (Campbell et al., 2019). Once the alarm call is played, hen behavior, particularly the latency to resume eating, is observed to measure the duration of vigilance (Anderson et al., 2021). Recently, the attention bias test was pharmacologically validated as a metric of anxiety in laying hens (Campbell et al., 2019) and broiler chickens (Lourenço da Silva et al., 2024). Research indicates a relationship between bird anxiety and their behavior in production environments. In free range housing, ISA Brown hens that spent more time in the indoor environment were more vigilant and took longer to resume feeding in the attention bias test compared to hens that spent more time in the outdoor range (Campbell et al., 2019). Similarly, Anderson et al. (2021) found that broilers housed in low-complexity environments resumed feeding faster and more birds resumed feeding overall in the attention bias test than those housed in complex environments. To our knowledge, the attention bias test has not been used to compare laying hen genetic strains, but a similar startle test demonstrates strain-specific responses to unexpected stimuli. A startle reflex response is elicited from sudden visual and auditory stimuli (Elston et al., 2000; Ross et al., 2019; Rentsch et al., 2024). Using a force plate to measure startle reflex amplitude in response to sudden light flashes, Lohman Brown Lite hens displayed a greater startle reflex amplitude than Lohman Selected Leghorn Lite hens (Rentsch et al., 2024). Elston et al. (2000) assessed latency to resume normal behavior after a variety of visual and auditory startle stimuli in two white strains. The authors found that Hy-Line W36 hens exhibited a longer latency to resume normal or pre-stimulus behaviors compared to Dekalb Delta layers (Elston et al., 2000).

In addition to behavioral responses in fearfulness and anxiety tests, stress physiology provides concurrent insight into the affective state of hens. Stress-induced hyperthermia occurs when the sympathetic-adrenal-medullary (SAM) axis triggers the release of adrenaline-associated hormones through the sympathetic nervous system, leading to vascular changes that direct blood flow towards highly vascularized areas of the body (Arfuso et al., 2022). These vascular changes can be evaluated by detecting variations in surface temperatures (Edgar et al., 2013). Infrared thermal imaging is a non-invasive method used to assess surface temperature in relation to vascular changes from stress-induced hyperthermia (Edgar et al., 2013; Nääs et al., 2014). Both the eye and comb are commonly used surface temperature measurements in chickens (Buijs et al., 2018; Edgar et al., 2013; Moe et al., 2012; Nicol et al., 2009), as the eye contains capillaries supported by the sympathetic nervous system (Arfuso et al., 2022) and the comb aids in thermoregulation (Moe et al., 2012). Previous research found that comb surface temperatures initially drop then rise to higher values than baseline after a handling stressor for laying hens, which is a temperature patten indicative of stress-induced hyperthermia (Edgar et al., 2013).

In cage-free environments, laying hens can express more fear behaviors if they are startled by sudden changes in light, sound, or movement from caretakers, which could have negative consequences for bird injuries and human-animal interactions. Comprehending how different genetic strains cope with sudden stressors may offer insights into their resilience in cage-free production systems. Therefore, the objective of this study was to determine if genetic strain influenced behavioral and physiological measures of fearfulness in an inversion test and an attention bias test. We hypothesized that genetic strain would influence cage-free laying hen responses behaviorally and physiologically. Specifically, we predicted that birds from two brown genetic strains (i.e., Hy-Line Brown and Bovan Brown) would be less fearful in an inversion test, less anxious in an attention bias test, and display lower comb and eye temperatures than birds from a white genetic strain (i.e., H&N White).

Materials and methods

This study was performed at North Carolina State University’s Poultry Teaching Unit and was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #22-219). Birds from three genetic strains were obtained from commercial cage-free pullet rearing facilities and transported to the university facilities. H&N White hens were reared in cage-free wire flooring until 11 weeks of age (WOA), while Bovan Brown and Hy-Line Brown hens were reared on litter flooring until 9 and 11 WOA, respectively. Upon arrival to university facilities, strains were distributed across nine floor pens (3.2m x 1.2m; n=3 pens/strain, 25 hens/pen). Each pen contained pine shavings on the floor for litter material (Tractor Supply Company, Brentwood, TN), one 1.75 kg feeder, one bell drinker, and wooden (perches 8.9″ of linear space/bird). Four nest boxes were added to all pens at approximately 17 WOA. Pullets were provided a DuMOR® 20% Chick Starter Crumble diet until switching to North Carolina State University’s standard layer diet at approximately 16 WOA. Feed and water were provided *ad libitum*. All hens were maintained on North Carolina State University’s layer lighting program (Anderson, 2018). Other research was conducted on the same flock to evaluate the effect of genetic strain on egg production and egg quality (Gulabrai et al., 2025).

Data collection

At the start of lay (22 WOA) and peak lay (32 WOA), 10 hens/pen were utilized for behavior testing (n=30 hens/strain/age). The week prior to testing, hens were randomly selected and leg banded for individual identification to ensure that the same individuals were used in both behavior tests at each age. Across ages, 25 of the 30 hens/strain were the same individuals tested at each age. The remaining 5 hens/strain were unique at each age as they were used for the terminal surface and core body temperature metrics described later. Two behavior tests were utilized – an attention bias test and an inversion test.

Attention bias test

The attention bias test chamber was a 16ft^3 chamber containing four nest pads on the floor, a feeder mounted to the wall, and blackout curtains lining the walls of the chamber. An identical chamber was also used during habituation to have two chambers habituating birds simultaneously. Hens were habituated to the testing chamber individually for 5 min/hen/day for three consecutive days prior to data collection. Feed and 5 live mealworms were placed in the feeder. Hens were considered habituated once they began eating from the feeder after being placed in the chamber, as opposed to displaying a freezing or exploratory response.

The attention bias test was performed during the first two consecutive days at each age. On testing days, the same chamber used during habituation was placed in a testing room that was visually isolated from other birds. A portable speaker (Sony XE200, Sony Corporation, Tokyo, Japan) was also placed in the chamber to play the auditory stimulus. A video camera (Sony ZV-1F, Sony Corporation, Bang Kadi, Thailand) was placed on a tripod in front of the chamber to record behavior. Hens were captured from their home pens, cooped in groups of eight to nine birds, and transferred to a holding room in the same barn as the home pens. The holding room was not visible from the testing room. One researcher was located inside of the testing room to take thermal images, and two researchers handled birds to bring them to the chamber. Researchers wore the same clothing throughout all testing days in order to limit the potential effects of novel clothing on hen response.

During testing, one bird was placed inside the test chamber facing the feeder. The testing room door was closed, and after 1 min, an auditory stimulus (450 Hz, 1 sec; wavtones.com) was played twice consecutively through the speaker via Bluetooth. Two minutes following the auditory stimulus, the bird was removed from the chamber and placed into another transport coop. Once all hens were tested, they were placed back into their home pens. The order of behavior testing within and across days was randomized and balanced by treatment.

Behavior data (Table 1) were coded from video recordings in BORIS software (v. 8.19.3) by one trained observer (inter-rater reliability between trainer and observer: 0.99, Cohen's kappa). The behavior definitions were adapted from Campbell et al. (2019), Anderson et al. (2021), and Lourenço da Silva et al. (2024), with modifications for the current study, and were coded according to these revised criteria. The frequency of head bobs was only recorded for 30 sec following the auditory stimulus as an indicator of vigilance behavior (Anderson et al., 2021; Campbell et al., 2019), and all other behaviors were recorded for 2 min post-stimulus.

Table 1
Definitions of laying hen behaviors observed in the attention bias test.

Behavior	Definition
Eating	Hen's head is level or below shoulders with head in feeder. Hen could be actively pecking feed or looking at feed in the feeder. Eating ends when the head is above shoulder level or head is at or below shoulder level but no longer in the feeder.
Head bob	Hen is making an exaggerated neck jerking movement. Only recorded for the first 30 seconds after startle stimulus has been played through the speaker.
Jumping out	Hen uses wings and/or feet to exit the chamber during the test. Hen is considered out of the chamber when both feet are past the threshold of the chamber.
Pecking	Hen's beak makes contact with an object in the chamber that is not the feeder (e.g., nest pads on floor, walls, or curtains).
Perching on curtain	Hen has both feet on the curtain shield at the front of the chamber.
Sitting	Hen's abdomen and legs are in contact with the ground.
Standing	Hen's body is supported by one or both legs. No other body parts are in contact with the ground.
Stepping	Hen has one foot in contact with the ground or curtain shield.
Vocalizations	Hen is emitting sound from its beak.

Inversion test

The inversion test was performed during the same weeks as the attention bias test on two consecutive days. One researcher handled birds to bring them to the testing room and pass them to the second researcher, who inverted the birds. As described above, researchers wore the same clothing throughout all behavior testing days to limit the effect of novel clothing and colors on hens' responses. Hens were captured from their home pens, cooped in groups of eight to nine birds, and transferred to the same holding room described for the attention bias test. The inversion test was performed in the same testing room as the attention bias test, which was not visible from the holding room.

During the test, a hen was passed to the researcher inside of the testing room, who immediately inverted the bird by both of its legs with one hand for 30 sec, following the methods described by Newberry and Blair (1993) and Archer and Mench (2014). Behavioral responses were video recorded (Sony ZV-1F, Sony Corporation, Bang Kadi, Thailand). After the test, the researcher reverted the bird back to its normal position and it was returned to another transport coop. Bird testing order was randomized and balanced by treatment within and across days to minimize potential time of day effects on response variables.

Behavior data (Table 2) were coded by one trained observer (inter-rater reliability between trainer and observer: 0.99, Cohen's kappa). The behavior definitions were adapted from Newberry and Blair (1993) and Archer and Mench (2014), with modifications for the current study, and were coded according to these revised criteria. Wing flapping count was observed at 0.25x speed to measure an accurate count. Wing flapping duration, vocalizations, and righting attempts were viewed at 1x (normal) speed. For wing flapping duration, the duration of each bout was recorded three times and then the average was recorded. All videos were viewed using the Google Drive media player.

Temperature changes

A FLIR E53 (Teledyne FLIR LLC, Tallinn, Estonia) thermal imaging camera was utilized to assess changes in comb and eye temperatures before and after behavior testing. Emissivity was set at 0.95, reflected and atmospheric temperatures were maintained between 19 to 21°C, and relative humidity remained at 50%. For the attention bias test, the hen was placed in the 16ft^3 chamber. A pre-stimulus image was taken, the auditory stimulus was played 60 sec later, and an image was taken at the time the auditory stimulus was played and every 30 sec for five minutes. For the inversion test, then was placed in the 16ft^3 chamber to capture a pre-test image. The hen was removed from the chamber, inverted for the test, and then the hen was immediately placed back in the chamber for to collect thermal images every 30 sec for 5 min. The chamber was lined with black curtains to create a color contrast, as backgrounds with lighter colors have the potential to cause false temperature readings. Images were analyzed using FLIR Thermal Studio Suite software (v. 2.0.21). The polygon measurement tool was used to outline combs and the spot tool was used to measure eye temperatures.

Core body temperatures (CBT) were assessed with ingested capsules (Anipill® capsule, BodyCAP, Hérouville-Saint-Clair, France). Five hens/strain were orally gavaged using a syringe and a 3/8″ OD Nalgene tube to administer the CBT capsules into the esophagus, ensuring passage to the

Table 2
Definitions of laying hen behaviors observed in the inversion test.

Behavior	Definition
Wing flapping	Both wings extending vertically then moving back toward the body in unison. The full movement from up to down establishes 1 wing flap.
Vocalizations	An audible sound emitted from the bird performing the test. Each individual sound is counted as one vocalization.
Righting	The head and trunk of a bird curling ventrally.

gizzard, one day prior to behavior testing at each age. Capsules recorded data every 1 min, with an accuracy of ± 1°C, throughout the duration of both fearfulness tests. Once testing was complete, all 15 hens were euthanized via cervical dislocation to obtain capsules from the gizzard at each age. Temperature data was then synchronized to monitors (Anilogger® Monitor, BodyCAP, Hérouville-Saint-Clair, France) and uploaded and summarized in corresponding software (Anilogger® Manager Software, BodyCAP, Hérouville-Saint-Clair, France).

Statistical analysis

Data were analyzed in R statistical software (version 4.2.0; R Core Team, 2021) using RStudio (version 2022.12.0+353) for macOS Sonoma 14.7.1. Generalized linear mixed model fits were assessed with the DHARMa package (version 0.4.7; Hartig, 2024), and linear mixed model fits were evaluated with the sjPlot package (version 2.8.16; Lüdecke, 2024). Models were selected based on meeting fit criteria and the lowest AIC value. For all analyses, the final models were obtained by a stepwise backward reduction using ANOVA for model comparison with a P value of > 0.05 as the criterion of exclusion. The significance of main effects was reported if their interaction terms were not significant. Model estimates, 95% confidence intervals, and Tukey's pairwise comparisons were obtained with the emmeans package (version 1.10.5, Lenth, 2024).

For all behavior models, genetic strain (factor with 3 levels: H&N White, Hy-Line Brown, Bovan Brown), age (factor with 2 levels: Start of Lay and Peak Lay), and their 2-way interaction were included as fixed effects, and individual bird ID nested in pen was included as a random effect. In the inversion test, generalized linear mixed models (glmmTMB package, version 1.1.10, Brooks et al., 2017) were used for the number of wing flaps (negative binomial distribution), wing flapping intensity (zero-inflated guassian distribution), the number of vocalizations (negative binomial distribution), and likelihood of hens righting (binomial distribution). A linear mixed model (nlme package, version 3.1.166, Pinheiro and Bates, 2000; Pinheiro et al., 2024) was used for the duration of wing flapping (square-root transformed). In the attention bias test, generalized linear mixed models (glmmTMB package, version 1.1.10, Brooks et al., 2017) were used for the likelihood of hens to resume eating and to peck (binomial distributions) and the number of head bobs and steps (negative binomial distributions).

For all thermal image models, separate models were run for start of lay and peak lay. For the inversion test, each model included genetic strain (factor with 3 levels: H&N White, Hy-Line Brown, Bovan Brown), time of test (factor with 11 levels: Baseline, 30 sec, 1 min, 1.5 min, 2 min, 2.5 min, 3 min, 3.5 min, 4 min, 4.5 min, and 5 min), and their 2-way interaction were included as fixed effects, and individual bird was included as a random effect. For the attention bias test, each model included genetic strain (factor with 3 levels: H&N White, Hy-Line Brown, Bovan Brown), time of test (factor with 12 levels: Baseline, Startle, 30 sec, 1 min, 1.5 min, 2 min, 2.5 min, 3 min, 3.5 min, 4 min, 4.5 min, and 5 min), and their 2-way interaction were included as fixed effects, and individual bird was included as a random effect. Linear mixed models (nlme package, version 3.1.166, Pinheiro and Bates, 2000; Pinheiro et al., 2024) were used for maximum comb temperature and average eye temperatures.

Similarly for all core body temperature models, separate models were run for start of lay and peak lay. For the inversion test, each model included genetic strain (factor with 3 levels: H&N White, Hy-Line Brown, Bovan Brown), time of test (factor with 6 levels: Baseline, 1 min, 2 min, 3 min, 4 min, and 5 min), and their 2-way interaction were included as fixed effects, and individual bird was included as a random effect. For the attention bias test, each model included genetic strain (factor with 3 levels: H&N White, Hy-Line Brown, Bovan Brown), time of test (factor with 12 levels: Baseline, Startle, 30 sec, 1 min, 1.5 min, 2 min, 2.5 min, 3 min, 3.5 min, 4 min, 4.5 min, and 5 min), and their 2-way interaction were included as fixed effects, and individual bird was included as a random effect.

Results

Inversion test behavior

Results for genetic strain and age effects on the inversion test are displayed in Table 3. Across ages, Hy-Line Brown hens performed more wing flaps (x^2=8.82, df=2, p=0.012) compared to the other two strains, whereas Bovan Brown hens exhibited more vocalizations (x^2=11.01, df=2, p=0.0041) than H&N White hens. Vocalizations were higher for all strains at the start of lay compared to peak lay (x^2=3.96, df=1, p=0.047). However, all strains displayed a significantly higher wing flapping intensity score at peak lay compared to the start of lay (x^2=5.17, df=1, p=0.023). There were no interactive effects of strain and age on any inversion test metric. The likelihood of hens righting, the duration of wing flapping, and wing flapping intensity were not affected by strain. The likelihood of hens righting, the duration of wing flapping, and the number of wing flaps were not different between ages.

Inversion test thermal images

Results for genetic strain and time of test effects on thermal image analysis for the inversion test are displayed in Tables 4, 5. At the start of lay, H&N White and Hy-Line Brown hens had significantly higher maximum comb temperatures compared to Bovan Browns (LR=17.66, p<0.0001). At peak lay, H&N White and Bovan Brown hens had significantly higher maximum comb temperatures than Hy-Line Brown hens (LR=7.20, p=0.027). Also at peak lay, maximum comb temperatures decreased in the first 30 seconds after inversion, then at 2.5 min post-test increased to temperatures higher than baseline (LR=75.21, p<0.0001). Test time did not affect maximum comb temperatures at the start of lay. Neither strain, test time, nor their interaction significantly affected average eye temperature at the start of lay or peak lay.

Inversion test core body temperatures

At the start of lay, a significant interaction between genetic strain and time of test was found for core body temperatures (LR=30.67, p<0.0001). H&N White and Bovan Brown hens had higher core body temperatures 4 and 5 min post-inversion compared to baseline and minutes 1 through 3 (H&N Whites, Baseline: 41.77, 1 min: 41.77, 2 min: 41.73, 3 min: 41.77, 4 min: 41.84, 5 min: 41.86 ± 0.071°C; Bovan

Table 3
The effects of genetic strain and age on laying hen behavior in an inversion test (mean±SEM)[1].

	Number of wing flaps	Duration of wing flapping (sec)	Wing flapping intensity (flaps/sec)	Likelihood of hens righting	Number of vocalizations
Genetic strain					
H&N White	8.36 ±2.57[a]	0.93 ±0.04	5.70 ±0.26	0.21±0.07	1.23±0.41[a]
Hy-Line Brown	21.27 ±5.49[b]	2.35 ±0.04	5.61 ±0.19	0.42±0.08	2.63±0.73[ab]
Bovan Brown	11.75 ±3.49[ab]	0.99 ±0.04	6.05 ±0.22	0.35±0.08	3.95±0.99[b]
Age					
Start of Lay	13.73 ±3.30	1.22 ±0.02	5.62 ±0.14[a]	0.28±0.06	2.89±0.66[a]
Peak Lay	12.62 ±2.89	1.49 ±0.02	5.96 ±0.15[b]	0.36±0.06	1.89±0.45[b]

[1] An inversion test was performed at the start of lay (22 WOA) and peak lay (32 WOA) by holding birds upside down for 30 sec and assessing behaviors from video recordings.

Table 4

The effects of genetic strain, age, and time of test on laying hen comb and eye temperature before and after an inversion test (mean±SEM)[1].

	Start of Lay		Peak Lay	
	Maximum comb temperature (°C)	Average eye temperature (°C)	Maximum comb temperature (°C)	Average eye temperature (°C)
Genetic strain				
H&N White	37.32±0.93[a]	33.84±0.50	39.76±0.19[a]	37.55±0.35
Hy-Line Brown	35.46±0.93[a]	33.10±0.49	39.08±0.19[b]	36.57±0.35
Bovan Brown	30.55±0.93[b]	32.50±0.49	39.68±0.19[ab]	36.69±0.34
Time of test				
Baseline	34.52±0.63	32.65±0.41	39.14±0.15[a]	36.44±0.30
30 sec	33.65±0.65	32.86±0.44	38.71±0.16[b]	36.43±0.30
1 min	34.13±0.65	32.96±0.42	39.21±0.15[a]	36.93±0.30
1.5 min	34.32±0.64	32.82±0.44	39.51±0.15[ac]	37.07±0.29
2 min	34.20±0.66	33.36±0.45	39.65±0.15[ac]	36.83±0.29
2.5 min	34.44±0.64	33.37±0.43	39.75±0.15[c]	36.77±0.29
3 min	34.31±0.64	32.95±0.46	39.69±0.15[c]	36.91±0.28
3.5 min	34.83±0.62	33.32±0.44	39.73±0.16[c]	37.25±0.33
4 min	34.52±0.69	33.77±0.50	39.73±0.16[c]	37.15±0.30
4.5 min	34.96±0.64	33.47±0.45	39.78±0.16[c]	37.13±0.30
5 min	35.00±0.66	33.07±0.50	39.68±0.16[c]	37.40±0.31

[1] An inversion test was performed at the start of lay (22 WOA) and peak lay (32 WOA) by holding birds upside down for 30 sec and assessing behaviors from video recordings. Thermal images were taken with a FLIR E53 camera and metrics include average maximum comb and average eye temperatures analyzed in FLIR Thermal Studio Suite software and measured in °C. The baseline image was taken before the inversion test was conducted. Following images were taken every 30 sec for a 5-min duration following the inversion test.

Browns, Baseline: 41.63, 1 min: 41.61, 2 min: 41.59, 3 min: 41.64, 4 min: 41.69, 5 min: 41.72 ± 0.071°C, mean ± SE). Hy-Line Brown core body temperature did not change throughout the test (Baseline: 41.59, 1 min: 41.59, 2 min: 41.57, 3 min: 41.58, 4 min: 41.61, 5 min: 41.61 ± 0.071°C). At peak lay, core body temperatures for all strains were highest 4 and 5 min post-inversion compared to all other times (LR=47.63, $p<0.0001$; Baseline: 41.72, 1 min: 41.77, 2 min: 41.78, 3 min: 41.81, 4 min: 41.82, 5 min: 41.84 ± 0.088°C). Strain did not affect core body temperature at peak lay.

Attention bias test behavior

Genetic strain and age results from the attention bias test are displayed in Table 3.5. There was a significant interaction between laying hen strain and age for the proportion of hens that resumed eating (x^2=8.18, df=2, p=0.017). H&N White hens were less likely to resume eating at peak lay compared to the start of lay. The opposite was true for brown strains, where Bovan Brown and Hy-Line Brown hens were more likely to resume eating as they aged. Genetic strain and age also had a significant interactive effect on the number of head bobs performed within the first 30 seconds after the startle stimulus (x^2=14.99, df=2, p=0.00056). H&N White hens performed the most head bobs at the start of lay and stayed consistent to peak lay. Hy-Line Brown and Bovan Brown strains increased head bobbing between start and peak lay. There was a significant interaction found between genetic strain and age for the likelihood of hens to peck. H&N White hens were less likely to peck as they aged, whereas Hy-Line Brown and Bovan Brown hens remained consistent between ages (x^2=6.91, df=2, p=0.032). There were no significant interactions or main effects of strain or age found for the number of vocalizations or steps exhibited.

Attention bias test thermal images

At the start of lay, significant interactions between genetic strain and time of test were found for average eye temperatures and maximum comb temperatures. At 2, 3, and 4 min following the auditory startle stimulus, H&N White hens had higher average eye temperatures than both brown strains, while Bovan Browns had the lowest average eye temperatures 4 min post startle (LR=44.21, p=0.0033; Figs. 1,2). For maximum comb temperatures, H&N White hens had higher temperatures from minutes 1.5 through 5 post-startle compared to Bovan Brown hens (LR: 53.21, $p<0.0001$; Fig. 3). Bovan Browns had the lowest maximum comb temperatures from 3.5 min through 4.5 min post-startle (Fig. 3). At peak lay, maximum comb temperatures were the lowest at baseline, then significantly increased by 30 seconds post-startle and remained high (LR=43.00, $p<0.0001$; Table 6). Finally, H&N White hens had higher average eye temperatures at peak lay compared to Bovan Browns (LR=7.30, p=0.026; Table 6). There were no significant differences found amongst strains at peak lay for maximum comb temperatures or amongst test times at peak lay for average eye temperatures.

Attention bias test core body temperatures

At the start of lay, H&N White hens had significantly higher core body temperatures than Hy-Line Brown hens (LR=6.40, p=0.041; H&N White, 41.99 ± 0.084°C; Hy-Line Brown, 41.69 ± 0.084°C, mean ± SE). Core body temperatures for all strains were significantly higher from 3 to 5 min following the startle stimulus compared to baseline, when startle stimulus was played, and minutes 1 and 2 (LR=101.50, $p<0.0001$; Baseline: 41.75, Startle: 41.75, 1 min: 41.79, 2 min: 41.84, 3 min: 41.89, 4 min: 41.91, 5 min: 41.94 ± 0.052°C). At peak lay, H&N White hens again had higher core body temperatures compared to Hy-Line Brown hens (LR=6.16, p=0.046; H&N White, 41.98 ± 0.089°C; Hy-Line Brown, 41.68 ± 0.080°C). Core body temperatures for all strains were higher than baseline by 2 min post-startle stimulus and remained high (LR=72.09, $p<0.0001$; Baseline: 41.77, Startle: 41.79, 1 min: 41.81, 2 min: 41.83, 3 min: 41.85, 4 min: 41.87, 5 min: 41.89 ± 0.049°C).

Table 5

The effects of genetic strain and age on laying hen behavior in an attention bias test (mean±SEM)[1].

		Likelihood of hens to resume eating	Number of head bobs	Likelihood to peck	Number of vocalizations	Number of steps
Genetic strain	**Age**					
H&N White	Start of Lay	0.61±0.10[a]	25.98±2.41[a]	0.43±0.10[a]	19.70±5.61	3.58±0.74
Hy-Line Brown	Start of Lay	0.31±0.09[b]	16.60±1.76[b]	0.29±0.09[ab]	14.84±4.49	3.73±0.76
Bovan Brown	Start of Lay	0.35±0.10[b]	13.62±1.54[b]	0.26±0.09[ab]	11.57±3.65	1.76±0.46
H&N White	Peak Lay	0.35±0.10[b]	24.40±2.32[a]	0.09±0.06[c]	12.52±4.12	2.78±0.63
Hy-Line Brown	Peak Lay	0.50±0.11[ab]	24.02±2.40[a]	0.20±0.08[bc]	12.86±4.06	4.49±0.93
Bovan Brown	Peak Lay	0.61±0.10[a]	23.32±2.29[a]	0.33±0.09[ab]	17.12±5.18	2.82±0.66

[1] An attention bias test was performed at the start of lay (22 WOA) and peak lay (32 WOA) by playing an auditory “startle” stimulus and assessing behaviors from video recordings for 30 sec after the stimulus for head bobs and 2 min after the stimulus for all other behaviors.

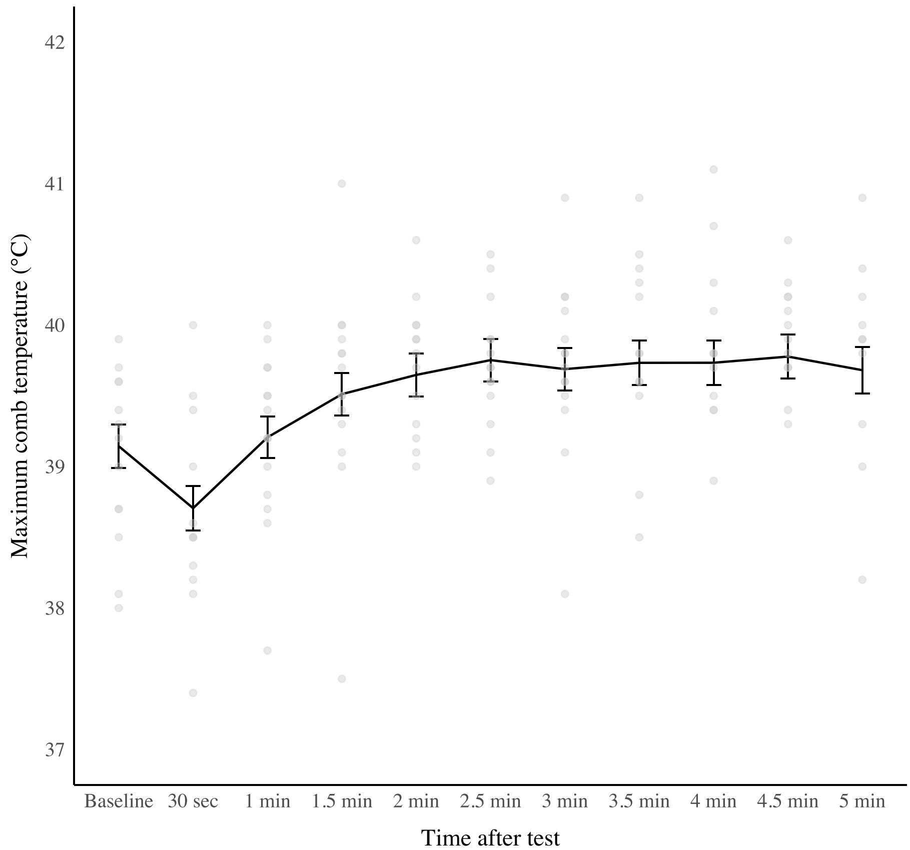

Fig. 1. The effect of time on maximum comb temperatures before and after an inversion test at peak lay[1]. The solid line represents the estimated mean values from the linear mixed effects model with SEM error bars. Raw data points are presented as grey dots.

[1] An inversion test was performed at peak lay (32 WOA) by holding birds upside down for 30 sec. Thermal images were taken with a FLIR E53 camera and maximum comb temperature was analyzed in FLIR Thermal Studio Suite software and measured in °C. The baseline image was taken before the inversion test was conducted. Following images were taken every 30 seconds for a 5-min duration following the inversion test

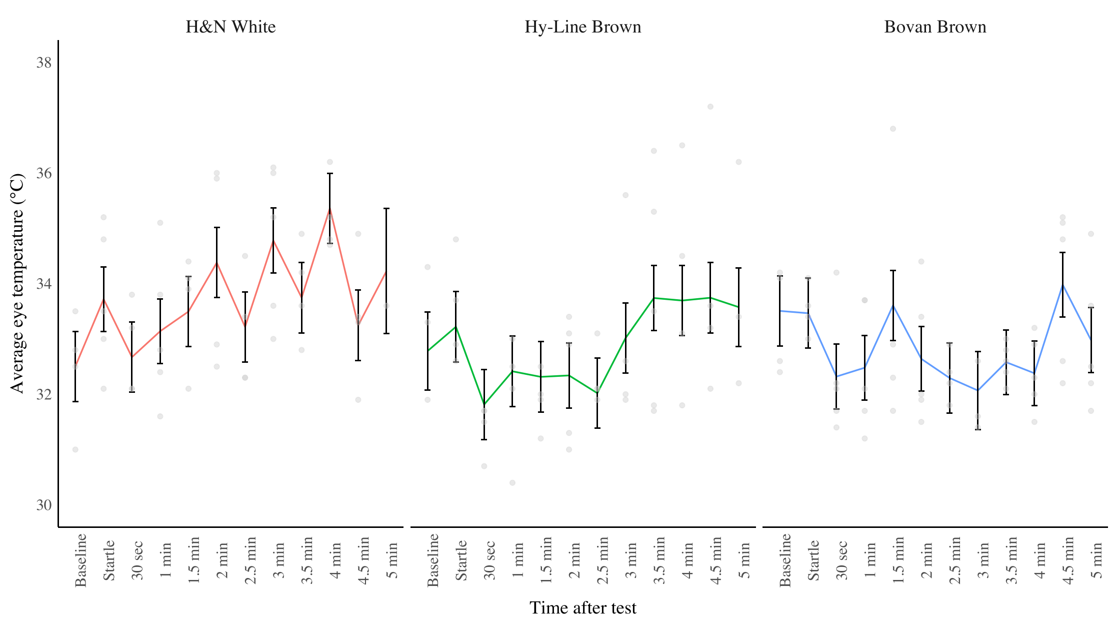

Fig. 2. Interaction of genetic strain and time on average eye temperatures before and after an auditory "startle" stimulus at the start of lay[1]. The solid line represents the estimated mean values from the linear mixed effects model with SEM error bars. Raw data points are presented as grey dots.

[1] An attention bias test was performed at the start of lay (22 WOA) by playing an auditory "startle" stimulus. Thermal images were taken with a FLIR E53 camera and average eye temperature was analyzed in FLIR Thermal Studio Suite software and measured in °C.

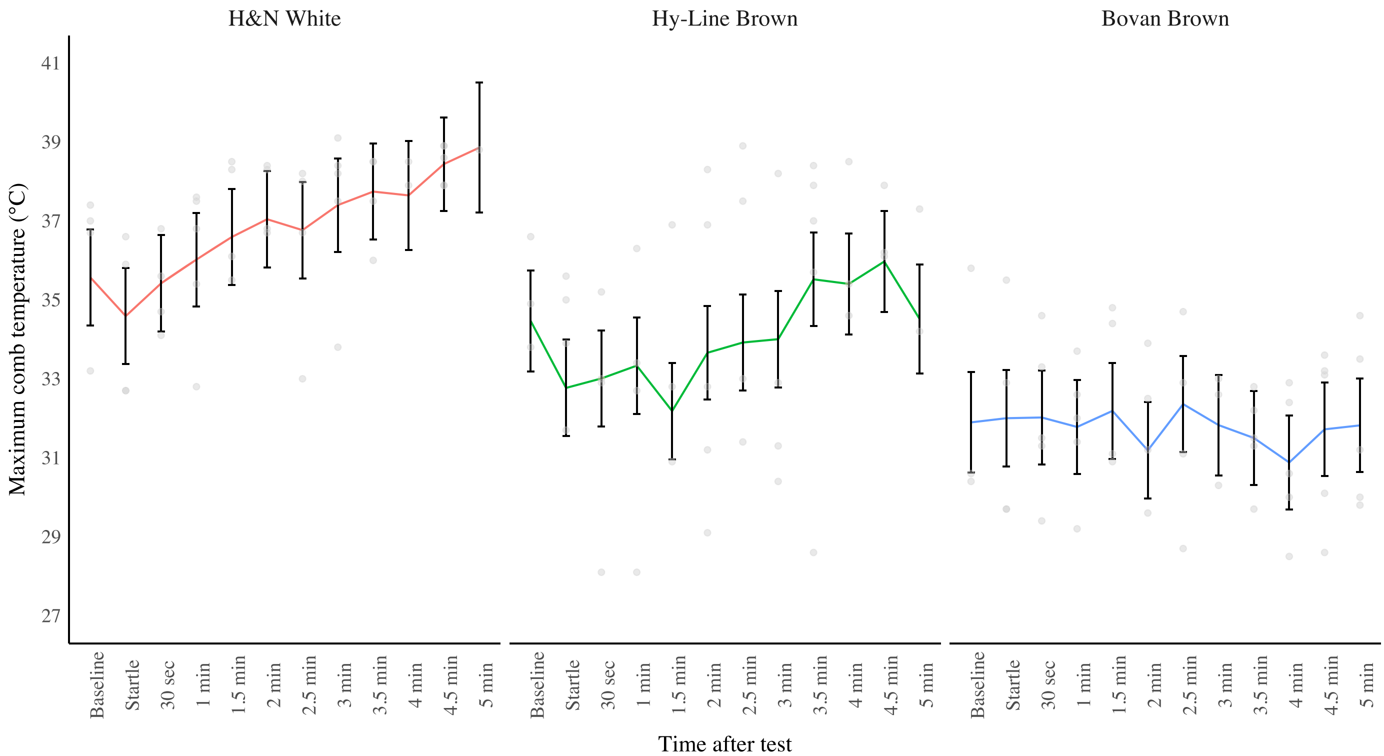

Fig. 3. Interaction of genetic strain and time on maximum comb temperatures before and after an auditory "startle" stimulus at the start of lay[1] . The solid line represents the estimated mean values from the linear mixed effects model with SEM error bars. Raw data points are presented as grey dots.
[1] An attention bias test was performed at the start of lay (22 WOA) by playing an auditory "startle" stimulus. Thermal images were taken with a FLIR E53 camera and maximum comb temperature was analyzed in FLIR Thermal Studio Suite software and measured in °C. The baseline image was taken before the attention bias test was conducted. "Startle" indicates the point at which the auditory stimulus was played. Following images were taken every 30 seconds for a 5-minute duration following the attention bias test.

Table 6
The effects of genetic strain and time of test on laying hen maximum comb and average eye temperatures before and after an auditory "startle" stimulus in an attention bias test at peak lay (mean±SEM)[1].

	Maximum comb temperature (°C)	Average eye temperature (°C)
Genetic strain		
H&N White	40.01±0.25	36.89±0.25[a]
Hy-Line Brown	39.65±0.25	36.18±0.21[ab]
Bovan Brown	39.44±0.25	36.10±0.21[b]
Time of test		
Baseline	38.99±0.20[a]	36.29±0.29
Startle	39.40±0.20[b]	36.38±0.32
30 sec	39.43±0.20[b]	36.04±0.29
1 min	39.67±0.18[b]	36.41±0.29
1.5 min	39.74±0.20[b]	36.47±0.29
2 min	39.71±0.20[b]	36.57±0.32
2.5 min	39.87±0.19[b]	36.38±0.30
3 min	39.92±0.19[b]	36.83±0.28
3.5 min	39.75±0.20[b]	36.18±0.32
4 min	39.93±0.20[b]	36.63±0.30
4.5 min	39.97±0.22[b]	36.57±0.32
5 min	40.00±0.24[b]	35.96±0.42

[1] An attention bias test was performed at peak lay (32 WOA) by playing an auditory "startle" stimulus. Thermal images were taken with a FLIR E53 camera and metrics include average maximum comb and average eye temperatures analyzed in FLIR Thermal Studio Suite software and measured in °C. The baseline image was taken before the attention bias test was conducted. "Startle" indicates the point at which the auditory stimulus was played. Following images were taken every 30 sec for a 5-min duration following the attention bias test.

Discussion

The objective of this study was to determine how laying hen genetic strains compared in their behavioral and physiological responses in two tests; an inversion test (fear) and an attention bias test (anxiety). Strains differed in their performance on the tests. H&N White hens were one of the least active strains when inverted and were the most likely to resume eating after being startled at the start of lay, initially suggesting less behavioral fear and anxiety. However, H&N White hens also had one of the highest maximum comb temperatures in both tests, the highest average eye temperature in the attention bias test, and one of the highest core body temperatures in both tests, suggesting that they experienced more stress-induced hyperthermia. They also displayed more vigilance behavior via head bobbing in the attention bias test. Both brown strains behaved similarly in the attention bias test and also had similar thermal values for their combs and eyes, although Bovan Brown hens displayed lower thermal values for some time points. In the inversion test, the two brown strains differed in wing flapping but there was no clear relationship with their thermal values.

Previous research also identified strain-specific behavioral differences in an inversion test, where Hy-Line Brown hens performed more wing flaps than White Leghorn strains, similar to the present study (Nelson et al., 2020). Brown strains also displayed a higher wing flapping intensity score compared to white strains (Brown et al., 2022; Nelson et al., 2020). In a startle reflex test, researchers reported that brown strains exhibited a higher startle amplitude, and therefore a stronger muscular "flight" response, than white strains after exposure to an unexpected light stimulus (Rentsch et al., 2024). Our study did not measure startle amplitude, but coupled with our results, brown strains have a more pronounced flight response. Furthermore, in the present study, hens from both brown strains were less likely to resume eating in the 2 min after the audio startle compared to H&N White hens at the start of lay. The latency to begin eating after an audio startle in the attention bias test is a validated metric of anxiety in chickens, such that a longer latency or recovery time is indicative of more anxiety (Lourenço da Silva et al., 2024). Taken together, these behavioral responses initially suggest that brown strains are more fearful and anxious than the white strain. However, this interpretation is complicated by H&N White hens performing more vigilance behavior in the attention bias test.

H&N White hens performed more head bobs at the start of lay compared to both brown strains and maintained a similar number of head bobs at peak lay. Head bobbing involves neck stretching and looking around, which is a component of vigilance behavior (i.e., alertness to a perceived threat) in response to sudden audio stimuli in laying hens and broiler chickens (Campbell et al., 2019; Anderson et al., 2021). Housing environment affected birds' vigilance behavior in attention bias testing in previous work (Campbell et al., 2019; Anderson et al., 2021). However, during a pharmacologic validation of the attention bias tests to assess anxiety in laying hens and broilers, vigilance behavior did not differ between birds given an anxiogenic drug and control birds (Campbell et al., 2019; Lourenço da Silva et al., 2024). Vigilance behavior may be a less reliable indicator of anxiety and affected by factors outside of the attention bias test, such as handling prior to the test. Previous research found that white strains of laying hens may be more affected by pre-test handling and consequently performed differently in a startle test than brown strains (Rentsch et al., 2024). If there is a relationship between vigilance and human-animal interactions, H&N White hens may have directed vigilance behavior towards the researcher in the testing room after the startle stimulus in the present study moreso than brown strains. Additional research is needed to fully understand the relationship between vigilance behavior and affective state.

H&N White hens mostly showed a greater autonomic response to stressors than both brown strains in both tests, which may indicate that they are experiencing more stress-induced hyperthermia and potentially more emotional stress (i.e., fear and anxiety) than brown strains. Stress-induced hyperthermia occurs from vascular changes activated by the sympathetic-adrenal-medullary (SAM) axis, where there is an initial decrease then rise in surface temperature in the onset of emotional stress (Edgar et al., 2013; Arfuso et al., 2022). Other researchers found that white strains of laying hens showed a larger decrease in comb temperature than brown strains immediately following a startle reflex test, but their thermal response did not correlate with their behavior in the test (i.e., startle amplitude; Rentsch et al., 2024). Similarly in the present study, the greater autonomic response from thermal images does not align with the reduced fear and anxiety behaviors in the tests. Since these physiological responses are not always accompanied by overt behavioral reactions, human caretakers face challenges in detecting stress in their flock through observation alone. These findings may indicate that the white hens displayed different behavioral coping strategies than brown hens while still experiencing stress, which has implications for the validity of interpreting behavioral outcomes from fear and anxiety tests alone.

White and brown strains exhibited different coping strategies in previous research as well, indicating that contexts and stimuli elicit strain-specific responses (Rentsch et al., 2024). For example, white strains exhibited more fearfulness through longer tonic immobility, while brown strains were considered more fearful with the novel object test (reviewed by Rentsch et al., 2023). Other authors have suggested that white strains are reactive while brown strains are proactive in their responses to fear (Pusch et al., 2018). Reactive responses are characterized by limited behavioral expression and increased physiological responses, whereas proactive responses are representative of increased behavioral activity and decreased autonomic responses (Pusch et al., 2018). Brown et al. (2022) and Nelson et al. (2020) also build upon this idea, concluding that white strains utilize more passive avoidance reactions to fear while brown strains use active avoidance strategies. Passive avoidance demonstrates the "freeze" response to fear, while active avoidance employs the "fight or flight" response to fear (Nelson et al., 2020; Brown et al., 2022). Overall, both brown strains demonstrated more active responses to the stressors evident in the inversion test and attention bias test compared to the white strain. H&N White hens also displayed higher surface and core body temperatures throughout testing. This passive approach indicates that while the white strain had a reduced behavioral response, they were still more physiologically responsive to the stressors present in both tests. The mechanisms in which hens utilize to cope with threats may provide better insight into how different strains are affected by various stimuli. Additionally, social dynamics in cage-free environments, such as hierarchies, competition, social learning, or social buffering, can influence stress responses and behavior in laying hens (Lay et al., 2011). The present study focused on individual hen responses, but future research should consider how social dynamics affect strain-specific fear responses in cage-free systems, such as piling behavior (Gray et al., 2020).

Both tests utilized in the present study were valid for inducing stress in laying hens as both induced autonomic response patterns, particularly at peak lay for both tests with thermal images and at both ages for core body temperature. Edgar et al. (2013) exposed chickens to a handling stressor and identified a thermal pattern in which surface temperatures initially drop below baseline, then steadily increase above baseline around 6 min after handling. Our research found a similar pattern and a faster response, such that maximum comb temperatures increased above baseline just 1 min after inversion, though this may have been influenced by prior handling. The Bovan Browns typically had the lowest surface temperatures, but are still within comb temperature ranges reported in previous research (Moe et al., 2012). Moe et al. (2012) proposed a potential correlation between reduced comb temperatures and pleasant experiences (e.g., anticipation/consumption of a reward) in laying hens. Based on our results, the Bovan Brown strain may not have been as physiologically affected by the stressors in both tests than other strains. Core body temperatures followed a similar pattern, such that there was a rise in temperature throughout the 5-min duration following the inversion test. However, core body temperatures require a rise in 0.5 to 1.5°C in order to be indicative of stress (Edgar et al., 2013) which was not evident in the present study. Measuring core body temperature for longer than 5 min may have provided more time to achieve this rise in temperature. Additionally, the internal body temperature of a chicken is around 40.5°C which all strains only slightly exceeded. To the author's knowledge, this is the first study to utilize core body temperature sensors in laying hens alongside thermal images to assess the autonomic response.

No clear pattern of strains acclimating between ages were found for either test, such that vocalizations decreased but wing flapping intensity increased, and opposite trends were found for strains between ages for the likelihood of hens to resume eating in the attention bias test. The absence of acclimation suggests that hens did not become less fearful overtime. Therefore, the stressors apparent in cage-free environments may elicit fear and anxiety responses through at least peak lay. Similarly, piling and smothering can be most prevalent in early and peak lay, where one of the causes is fear (Gray et al., 2020). However, it is evident that the mechanisms in which hens cope with these stressors vary between genetic strains and should be considered when housing them in cage-free production systems.

Conclusion

Hy-Line Brown and Bovan Brown strains demonstrated more behavioral responses of fear and anxiety in both tests, while the H&N White strain demonstrated more physiological responses of emotion distress. These findings align with previous research that describes brown strains as utilizing active avoidance strategies to cope with fear and stressors, while white strains demonstrate passive avoidance mechanisms (Brown et al., 2022; Nelson et al., 2020). Despite showing a limited behavioral response, the H&N White hens were still physiologically reactive in both tests. These findings have implications for interpreting future fear and anxiety tests, such that behavioral metrics should be coupled with physiological metrics for a comprehensive view of how the birds are coping. It is important to acknowledge that the method in which hens use to cope does not necessarily correlate with the effectiveness of the coping style, although suitable coping mechanisms can benefit animal welfare (Colditz and Hine, 2016). Recognizing how various genetic strains of laying hens cope with challenges may allow producers to gain a thorough understanding of how management practices impact the welfare of laying hens. Therefore, the findings of this study may aid producers in selecting genetic strains best suited for their particular cage-free environment.

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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