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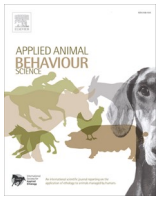

# Brown and white layer pullet hybrids show different fear responses towards humans, but what role does light during incubation play in that?

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ABSTRACT

Good early life conditions are increasingly recognized as essential to animal welfare later in life. The use of light during incubation might improve coping capacities and welfare in later life in poultry, by more closely approximating chicken natural environments compared to the current conventional incubation in darkness. Previous studies showed that lighted incubation resulted in more lateralized chicks, a more pronounced daily behavior rhythm, earlier onset of melatonin rhythms, and lower stress reactions to various stressors after hatching. Most existing research, however, has been conducted on broilers, and little information on lighted incubation is available for laying hens. In the current research, Dekalb White and ISA Brown eggs were incubated in complete darkness or in a cycle of green 12 L:12D throughout incubation, and five fear of human tests were performed on the 387 chickens during the rearing phase. We expected dark-incubated chickens to show stronger fear responses than light-incubated chickens. That was only the case for one of 15 behavior measurements taken during the tests ($p < 0.05$). In addition, white layer hybrids are known to be flightier and more fearful than brown hybrids. In this study, white chickens indeed showed stronger fear responses than brown chickens in 12 of the 15 behavior measurements ($p \leq 0.002$). Furthermore, we expected light during incubation to have stronger effects on white chickens than on brown chickens, because of the stronger transmission of light through white eggshells. However, the interaction between hybrid and incubation was never significant ($p \geq 0.18$). Finally, contrary to our expectations, there was no effect of the incubation treatments or the hybrid on plasma corticosterone responses to a manual restraint test ($p \geq 0.36$). Since there was a hybrid effect on behavior in this test, it is reasonable to think that behavior reflected coping style, rather than fear level. To conclude, the light regime used in this study does not seem as promising as expected to improve laying hen welfare. Finally, the brown hybrid was usually less fearful than the white hybrid, though there were some exceptions depending on the stressor, and that should be taken into account in research and in laying hen management.

## 1. Introduction

Poultry eggs are typically incubated in complete darkness. In nature, however, the hen occasionally leaves the nest (Archer and Mench, 2014a; Mrosovsky and Sherry, 1980), and the eggs are exposed to some light during the day. Positive effects of light during incubation have been reported for broiler chicken welfare (Archer, 2017; Archer and Mench, 2017, 2014b, 2014a, 2013).

Several studies (Archer, 2017; Archer and Mench, 2017, 2014b, 2014a, 2013) demonstrated that broilers incubated with light showed more species-specific behaviors, more pronounced physiological rhythms and lower stress reactions to different stressors. Namely, light-incubated chickens, as compared to dark-incubated ones, were more active at the beginning of the day and less active at night, more lateralized in their behavior, and already had a daily melatonin rhythm at hatching. Finally, they had a lower plasma corticosterone response to being isolated for one hour in a crate, and less fearful responses in various fear tests (tonic immobility, emergence, inversion, approach and

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isolation tests).

The few studies that have investigated light during incubation in laying hens reported positive effects on their welfare as well. Light-incubated layer chicks displayed less severe feather pecking behavior (Özkan et al., 2022) and performed better in a cognitive task (transitive inference) (Daisley et al., 2010) compared to dark-incubated chicks.

Two mechanisms can explain those positive effects. One of the mechanisms involved is brain lateralization (Rogers, 1982). Because of the embryo's position in the egg, the two brain hemispheres receive different light exposure. As a result, they specialize in different functions (Archer and Mench, 2014a), and develop an appropriate stress-coping strategy. Another possible mechanism involves hormones. Melatonin is a light-sensitive hormone and is greatly involved in behavioral and physiological development in birds (Nichelmann et al., 1999). Its stimulation during avian embryogenesis could thus influence animal welfare later in life (Saito et al., 2005).

Light properties, such as exposure duration, cycle pattern, and wavelength (color), can result in different effects (Archer, 2017; Archer et al., 2009; Archer and Mench, 2014b, 2013; Erwin et al., 1971; Rogers, 1982). A cycle of 12L:12D during the entire incubation (21 days) seems the most promising to improve chicken welfare.

Results regarding the effects of wavelength are less univocal. On the one hand, red (630 nm) and white light, but not green light (520 nm) during incubation decreased fearfulness in broilers compared to dark incubation (Archer, 2017). On the other hand, only green light (518 nm), but not white light (442 nm) during incubation reduced severe feather pecking behavior in later life of laying hens (Özkan et al., 2022). This seems to indicate promising effects of green light on laying hens.

White laying hens are known to be more fearful than brown laying hens (*e.g.* Campo and Dávila, 2002; De Haas et al., 2014a; Fraisse and Cockrem, 2006; Jones and Faure, 1981a; Mahboub et al., 2010; Uitdehaag et al., 2008a). However, despite being among the most commonly used hybrids, Dekalb White and ISA Brown have not been compared to each other in previous studies on fear responses. Since differences also exist between white hybrids and between brown hybrids (Albentosa et al., 2003), it is worth investigating these specific hybrids.

In addition, light during incubation might affect white and brown laying hens differently, as light transmission through Dekalb White's white eggshells is 5.5 times higher compared to ISA Brown's brown eggshells (Manet et al., 2023). This raises the question whether green light is indeed beneficial to all layer hybrids.

In the present study, Dekalb White and ISA Brown eggs were therefore incubated either in a cycle of 12L:12D with green LED light throughout incubation, or in complete darkness as a control. After hatching, and throughout the rearing phase, different fear of humans measurements were taken to assess whether light during incubation affected both hybrids, potentially in different ways. In addition, hybrid differences in fear responses were investigated. Several aspects of fear of humans were measured on individual or group level.

Light during incubation was expected to lower fear and corticosterone responses compared to dark incubation. In addition, we expected Dekalb White chickens to show more fearful behavior and higher corticosterone levels compared to ISA Brown chickens. Finally, light being transmitted more through white *versus* brown eggs, we expected stronger effects of lighted incubation on the Dekalb White chickens than on the ISA Brown chickens.

## 2. Materials and methods

### 2.2. Animals

Eggs from ISA Brown and Dekalb White laying hens were provided by Hendrix Genetics through the hatchery Het Anker (Ochten, the Netherlands). They came from middle-aged parental flocks (between 37 and 50 weeks of age), housed in traditional single-tier housing.

### 2.3. Incubation and hatching procedure

For practical reasons, the experiment was performed in two rounds. In each round, 600 eggs (300 of each hybrid) were incubated at Wageningen University and Research (WUR), the Netherlands. They were equally and randomly distributed over four incubators. Two of them were HatchTech incubators each with a setting capacity of 1400 eggs (HatchTech, Veenendaal, the Netherlands). The other two were climate respiration chambers each with a setting capacity of 400 eggs (Verstegen et al., 1987). For more information about the four incubators, see (Güz et al., 2021).

During the first 18 days of incubation, the eggs were gradually turned every hour at an angle of 60°, and the average relative humidity and temperature were 57.5% and 37.8 °C, respectively. From embryonic day (ED) 19, the egg-turning stopped, and the average relative humidity and temperature were 58.5% and 36.3 °C.

In two of the incubators (one of each type), green (520 nm) LED-strips (Barthelme Y51515213 182007 LED strip) were installed so that the light intensity was around 400 lux at egg level. The eggs were exposed to a cycle of green 12L:12D throughout incubation. The other two incubators were kept in complete darkness. The standard design was therefore a 2 × 2 factorial arrangement (hybrid type x incubation treatment) repeated in two rounds.

At ED19, eggs were moved to hatching baskets. From then on, and until ED21, hatching and health checks were performed on new hatchlings every six hours. Male chicks were killed by cervical dislocation. Neck-tags with unique numbers were given to the female chicks for identification throughout the experiment. The females were then placed back in the hatching baskets. On ED21, all hatched female chicks were transported in cardboard boxes from WUR to their rearing facility at the Faculty of Veterinary Medicine of Utrecht University (UU) by a professional poultry transporter.

### 2.4. Husbandry

Groups of 10 chickens from the same hybrid and same incubation treatment were housed in 20 pens of 246 * 88 $cm^2$ and 241 cm high. The pens were separated by a wire-mesh fence and a 61 cm high wooden partition blocking the view to neighboring pens on that height. The closed concrete floor was covered with wood shavings.

The light regime, temperature and humidity followed the ISA Brown and Dekalb White rearing guidelines (Hendrix-Genetics, 2020a, 2020b). More specifically, non-flickering dimmable bird-friendly lamps (GlassLux Standard 1×36W Philips IP67 colour 830, Boon Agro) were used. In addition, the chickens were exposed to natural daylight through four ceiling windows, with automatic blinds that were opened and closed at fixed hours throughout the experiment to avoid a season effect on the (natural) light exposure. The four treatment groups were semi-randomly distributed over the 20 pens, taking into account the location of the ceiling windows.

The chickens had *ad libitum* access to feed ("Starter I" from De Heus until 6 weeks old, and thereafter "Start & grow" from Havens). It was provided in a large disk-shaped feeder put on the floor first, then in a hanging food dispenser from 9 days old, with the height adjusted as the chickens grew. Water was supplied from a hanging dispenser with three nipples and cups.

Until the chickens were 5 weeks old, the rearing environment was enriched with commercial chick brooder heating plates of 25 * 25 $cm^2$ (WP-25 from Kleinveeservice). Brooder plates are used to mimic the presence of a mother by offering a warm and dark shelter. Their height was adjusted as the chickens grew. Black cotton fabric was cut and placed around the brooder plates as curtains with multiple openings to provide a dark shelter during the first week. However, the chicks were getting stuck with their neck-tags in round 1, so the curtains were not used in round 2. Wooden perches were provided on the floor until 6 weeks old, thereafter replaced by a higher (61 cm high) wooden

platform on which two 88 cm-long plastic mushroom-shape perches were fixed (15 cm higher). In addition, a classical music radio station (Dutch station NPO4) played 24/7, as it has been proven to reduce stress levels by habituating animals to human voices and attenuating the abruptness of sudden loud noises in the facility (Davila et al., 2011; De Haas et al., 2014a).

Welfare of the chickens was monitored on a daily basis: a handful of grains were provided in each pen every morning to spot weak or irresponsive individuals. In addition, a thorough check was performed on a weekly basis (twice a week the first two weeks of life), involving handling and weighing of all 200 individuals. Finally, chicks were sprayed with Newcastle Disease vaccines (Nobilis® ND Clone 30, MSD) on days 14, 68 and 95.

Additional handling that the chickens were exposed to during the experiment were the application of 1) light-weight numbered backpacks at 9 weeks old to ease the identification and to shorten the catching process, and of 2) leg bands with RFID tags from 5 to 12 days old, from 6 to 9 weeks old, and from 19 to 24 weeks old. Finally, in round 1, three Dekalb White males were identified (sexing errors) and removed at 5 weeks old from three different pens.

### 2.5. Behavior and physiological measurements

In total, five human-fear tests (one of them including a physiological measurement) were performed on the chickens to measure different aspects of fear of humans (Table 1). Each of the tests was performed on all of the chickens, and the chicken testing order was randomized prior to each test using an online randomizer. The detailed protocols are available at the following address: https://doi.org/10.5281/zenodo.8325446.

When the tests were performed on the individual level, several experimenters performed the tests over the days and rounds. In each test, the number of chickens tested by each experimenter was balanced per treatment group to avoid any confounding experimenter effects. The individual tests were performed in one or two testing rooms a few meters away from the room of the home pens, in the same building. The same bird-friendly lamps as in the home pens were present in the testing rooms.

The chickens underwent other tests, but those are not reported in the present paper (Table 1). No additional physiological measurements were taken from the chickens.

#### 2.5.1. Voluntary approach test

The voluntary approach test (VAT) was performed to measure the chickens' individual reactions to a familiar human (Hewlett and Nordquist, 2019). A familiar food reward was used in the hope to counter-balance the effect of social isolation on the chicken's motivation to approach the human and to include a positive aspect to the test.

The VAT was performed twice on each chicken: at 6 and at 16 weeks old (respectively VAT1 and VAT2). The number of chickens tested in each round was, respectively, $N_{R1} = 189$ and $N_{R2} = 198$. In each round, though, one chicken reached a humane end point before the VAT2 and was therefore only tested once.

Maximum one week before the start of the test, the chickens were habituated to dry mealworms (the food reward). A handful of mealworms was scattered in their home pens, and the habituation was considered successful when at least two chickens of each pen had pecked at a mealworm while the experimenter was still in the pen.

A chicken was collected from its home pen and taken to the testing room in a transport box. It was placed in the top-left corner of the test arena and given one minute of habituation, during which it could see and access a handful of its regular feed in the center of the arena. After the habituation, a familiar human (*i.e.* a researcher or student who had already spent time in the stables to weigh the chickens and/or performed another behavior test) entered the arena and kneeled down in the bottom-right corner of the test arena. If the chicken had moved during habituation, the experimenter's location did not change. If the chicken was at the bottom-right corner, it was gently moved aside so the experimenter could take position. The experimenter placed two dry mealworms on the feed and kept three more on their hand, which was placed next to the feed throughout the test. The test ended when the chicken pecked at a mealworm, the feed or the experimenter, or after two minutes – whichever occurred first.

Three parameters were scored: the movement of the chicken during the test, the latency to approach the human, and the latency to peck. The first two were scored from the video recordings, while the last one was scored live by the experimenter themselves.

The movements of the chicken were categorized as (from the most to the least fearful): immobile (the chicken remained at the same position throughout the test; head movements were still considered as immobile), walking around (the chicken made at least one step towards the left or the right during the test) or walking straight to the feed (the chicken walked on a straight line from its initial position to the feed; the movement was still considered straight if the test finished before the chicken pecked).

The chicken was considered to have approached the experimenter if its head and neck were within a one-chicken distance from the experimenter. The chicken was considered to have pecked if it had pecked at a mealworm, the feed, of any body part of the experimenter (including clothes and stopwatch). Short latencies to approach or to peck were associated with low fearfulness.

#### 2.5.2. Human approach test

The human approach (HA) test was performed to measure the groups' fear responses to a familiar human in the chickens' home pens, a situation easily translatable to practice.

The human approach test took place when the chickens were 10 weeks old and was performed on group level by the same familiar experimenter for both rounds in all 20 pens.

The protocol was adapted from an experiment on pig behavior (Marchant-Forde et al., 2003). The experimenter entered the home pen and stood in the bottom-right corner for five minutes. During those five minutes, the latencies of the first three chickens to approach (*i.e.* making at least one step towards the experimenter) and to touch the experimenter (*i.e.* touching any body part including the shoes, with their beak, feet or any body part) were recorded. Short latencies were associated with low fearfulness.

After five minutes, the experimenter crouched down and performed a capture simulation (CS). The experimenter stretched their right arm and pretended to reach for a chicken, not targeting any specifically, and slowly moved their arm from side to side. The first reaction of each chicken was scored (from the most to the least fearful): fleeing (making

**Table 1**
Chronology of the behavior tests performed during the rearing phase, with the age of the chickens given in weeks. The tests in bold are the tests included in this article, and for these the sample sizes are given.

| Age (w) | 0 | 1 | 2 | 3–4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12–13 | 14–15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tests[a] | | NOT, NET | | LT | FPO | **VAT1** | | OF | | NOT2, **HA**, FPO | **TI** | | **MR** | | **VAT2** |
| Chickens tested (#) | | | | | | **387** | | | | **386** | **385** | | **385** | | **385** |

[a] NOT1 = Novel Object Test. NET = Novel Environment Test. LT = Lateralization Test. FPO = Feather Pecking Observations. VAT1 = Voluntary Approach Test 1. OFT = Open Field test. HA = Human Approach test. TI = Tonic Immobility test. MR = Manual Restraint test. VAT2 = Voluntary Approach Test 2

at least one step in the opposite direction of the experimenter), remaining immobile (not making any step in any direction, but could make body or head movements), approaching (see the definition for latency to approach), or touching (see the definition for latency to touch). The test ended after the CS. All the pens were tested on the same day.

In round 1, an unforeseen inspection took place prior to the testing. Therefore, the test was delayed, and a similar situation was mimicked during round 2.

### 2.5.3. Tonic immobility test

Tonic immobility is an anti-predator strategy naturally expressed in chickens, which can also be induced by placing the chicken on its back. The tonic immobility (TI) test is a standard test to investigate intrinsic fearfulness in chickens (Jones and Faure, 1981a). Though not directly a human fear test, the TI test was included in this paper because of the strong human-chicken interaction required for it (namely, catching, handling and inducing TI).

The TI test was performed on the individual level when the chickens were 10 weeks old. The test was performed on all chickens in the experiment ($N_{R1} = 188$, $N_{R2} = 197$) over three to four days.

A chicken was collected from its home pen and taken to an unfamiliar room, adjacent to the stables, in a transport box. In the unfamiliar room, the TI cradle was on a 76-cm-high table. The chicken was taken out of the transport box and placed on the cradle in a standardized way: the experimenter placed their right hand on the breast of the chicken, their left hand on its back, and flipped the chicken around in a supine position (Jones and Faure, 1981a; van der Eijk et al., 2018). They placed the chicken on the cradle with the neck at the end of the cradle to allow the head to hang, slowly removed their left hand and, with it, gently guided the head downwards while covering its eyes. The chicken was restrained in this position for 10 s, after which the experimenter removed their hands. Another 10 s were counted, during which, if the TI ended, the procedure was restarted with a maximum of three inductions. If not, the stopwatch was started to measure the TI duration. The TI ended when the chicken rose back on its legs, or started struggling (rolling on its side and/or flapping its wings) to rise up. The test ended at the end of the TI or after five minutes – whichever came first. Short TI duration and high number of inductions were associated with low fearfulness.

### 2.5.4. Manual restraint test and blood sampling

The manual restraint (MR) test measures an individual chickens' fear responses to a negative interaction with a human: a five-minutes restraint. Because this test is a commonly accepted negative experience for the chickens, it is usually accompanied with blood sampling to measure the physiological stress response through plasma corticosterone, which was also done in this research.

The MR test was performed individually on all chickens ($N_{R1} = 188$, $N_{R2} = 197$) when they were 14–15 weeks old. The test was performed over 8–9 working days (10–11 days total).

*2.5.4.1. Manual restraint test.* A chicken was transported to a test room, away from the stables, where an experimenter would proceed to the MR test: they placed the chicken on a cardboard-covered table on its left side, their left hand on its body applying gentle but firm pressure, and their right hand loosely holding the legs. The MR lasted for five minutes, during which the latency to struggle, the number of struggles, the latency to vocalize and the number of vocalizations were recorded (Bolhuis et al., 2009; van der Eijk et al., 2019). After that, the chicken was transported back to the stables.

Short latencies to struggle and vocalize, and a high number of struggles and vocalizations, were associated with low fearfulness.

There were two test rooms for the MR. The treatments were equally distributed across test rooms to counter potential room effects. Normal room light, rather than bird-friendly lamps, were used in an attempt to make the environment even more stressful.

*2.5.4.2. Blood sampling.* Blood samples were taken from the wing veins of the chickens during the MR test. Around half of the chickens of each round underwent three blood samplings, while the others underwent only one. A baseline sample was drawn within three minutes after the experimenter entered the home pen to catch the chicken ($N_{R1} = 83$, $N_{R2} = 95$) (Ericsson et al., 2014); a peak sample was drawn 15 min after the beginning of the acute stress (namely, the manual restraint) ($N_{R1} = 184$, $N_{R2} = 190$) (van der Eijk et al., 2019); and a recovery sample was drawn 30 min after the beginning of the manual restraint ($N_{R1} = 85$, $N_{R2} = 89$) (Fraisse and Cockrem, 2006). More specifically, the earliest and latest baseline samples were taken 56 and 175 s after catching, resulting in a gap of 119 s (1′59). The gap between the earliest and latest peak sampling times was of 210 s (3′30). The gap between the earliest and latest recovery sampling times was of 225 s (3′45). Between the samples, the chickens were kept in transport boxes next to each other in the same room as the home pens, and holes in the boxes allowed them to see and hear the other chickens.

Blood was collected from the wing veins: a 27 G needle (Braun Sterican Hypodermic Needles) was used to puncture the vein, immediately after which blood was collected in heparin-coated capillaries (Hirschmann, Eberstadt, Germany). The blood was blown out of the capillaries using a pipette bulb (Hirschmann, Eberstadt, Germany) and stored in Eppendorf tubes on ice for a maximum of 3 h until centrifugation (VWR Avantor Amsterdam Nederland, 521–1647, Micro Star 17 R). The plasma was then pipetted out and stored at − 20 °C until analysis.

For practical reasons, one to three different experimenters performed the blood sampling on the same chicken. In addition, the chickens that underwent three samples were tested in the first room, and the others in the second one (see description in the previous subsection).

The testing order was planned to ensure a time gap between two chickens from the same pen, to avoid a rise in corticosterone due to the capture process of the preceding pen mate. Finally, corticosterone having a circadian rhythm, the testing order also involved a loop between the four treatment groups (*e.g.* Brown-Dark, White-Dark, Brown-light, White-Light).

*2.5.4.3. Plasma analysis.* Corticosterone was extracted from the plasma with methanol, and the extracts were analyzed in an ELISA with a kit from Cayman chemical (501320 Corticosterone ELISA kit). The samples were analyzed in duplicates.

For each sample, the coefficient of variation (CV%) between replicates was calculated. If the CV% was higher than 20%, it was considered too high and the sample was analyzed again, leading to a maximum of six replicates. When required, the most divergent replicates were removed from the analyses and the average of the remaining replicates were added to the dataset.

Low corticosterone levels were associated with low stress responsiveness.

### 2.6. Statistical analyses

All the analyses were performed using RStudio Desktop 2022.07.1 + 554.

The models used and their distribution are shown in Table 2. For survival analyses, the distribution was chosen based on the model giving the lowest AIC. For the movement during the VAT, a Bayesian model was used, as it allowed a 2-by-2 comparison of the three movement categories while also investigating the effect of the incubation treatment and hybrid in each of the categories, which would have not been possible in another model.

The models were all first built with the hybrid, the incubation

**Table 2**
Statistical models used to analyze each parameter. VAT = voluntary approach test. HA = human approach test. TI = tonic immobility test. MR = manual restraint test. (1) = for the test performed at 6 weeks old. (2) = for the test performed at 16 weeks old. [A] 4 analyzes were performed: number of individuals that (1) fled, (2) remained immobile, (3) approached or (4) touched. [B] 3 analyzes were performed: number of individuals that needed 1, 2 or 3 inductions.

| Behavior test | Parameter | Model | Distribution |
|---|---|---|---|
| VAT | Movement during the test | Bayesian generalized linear multivariate multilevel model | Categorical |
| | Latency to approach | Survival analysis | Logistic (1) Gaussian (2) |
| | Latency to peck | Survival analysis | Gaussian |
| HA | Latency to approach | Survival analysis | Logistic |
| | Latency to touch | Survival analysis | Gaussian |
| | Reaction to capture simulation | Generalized linear mixed model | Poisson[A] |
| TI | Latency to rise | Survival analysis | Logistic |
| | Number of inductions | Generalized linear mixed model | Poisson[B] |
| MR | Latency to struggle | Survival analysis | Gaussian |
| | Number of struggles | Generalized linear mixed model | Poisson |
| | Corticosterone levels | Generalized linear mixed model | Gaussian |

treatment, their two-way interaction, and the round as fixed factors. Experimenter was also added as a fixed factor for all parameters, except for the HA test, which was performed by the same person. The experimenter was also excluded from the model for the number of inductions in the TI test, as the data would otherwise only range from 0 to 6 (median (Q1; Q3) = 1 (1; 2)) and could not be analyzed. Without the experimenter, the data ranged from 0 to 10 (median (Q1; Q3) = 3 (1; 7)). For the peak and recovery corticosterone levels during the MR test, the experimenter performing the MR was included, rather than the one drawing the blood sample, as the goal was to measure the corticosterone response to the MR.

Though the observational unit was the individual chicken, the pen was corrected for as a random effect. In the case of the human approach test, the pen was the observational unit.

Since corticosterone has a circadian rhythm, and can change with age especially around puberty (Kiezun et al., 2015; Webb and Mashaly, 1985), the age of the individual chickens (in weeks) and the time of blood collection were also corrected for as random effects in the model.

When the interaction was not significant, it was removed from the models.

For the main effects, only the outcome from the final models are hereafter reported. For the interactions, the outcomes from the last model including them are reported. The estimate (est), the 95% confidence interval (95% CI) and, when available, the *p*-values are reported. An effect was considered significant if the 95% CI did not include 0 (and if $p < 0.05$). In the case of the Bayesian model, the statistics software did not provide *p*-values, so those are omitted.

If any analysis showed a significant experimenter effect, this was investigated further by performing a pairwise Wilcoxon rank sum post-hoc test. This test was made simpler by only including the experimenter as a fixed factor. The *p*-values were corrected for multiple testing with the Bonferroni method, *i.e.* by multiplying them by the number of comparisons. When more than two experimenters were compared, the significant p-values are reported in the results for each measurement.

Wilcoxon tests for paired data were performed to compare the different blood samples to each other (baseline *vs.* peak, peak *vs.* recovery, and baseline *vs.* recovery) within each treatment group.

For analyses, corticosterone values were calculated by subtracting the baseline from the peak (for the "peak") and the peak from the recovery sample (for the "recovery"). They will be referred to as "peak" and "recovery" for easy reading.

Analyses were also performed on the raw values, but the outcome remained the same (data not shown) except for a hybrid effect on the non-transformed values of the recovery level (est = −37.67, 95% CI = [−63.049; −10.66], $p = 0.006$).

## 3. Results

The datasets are available at the following address: https://doi.org/10.5281/zenodo.8325446.

### 3.1. Voluntary approach test

#### 3.1.1. Movement during the test

The number of chickens performing each movement was analyzed. The incubation treatment influenced the chickens' movement at 6 weeks old (Fig. 1): light-incubated chickens moved around more compared to dark-incubated chickens, which remained immobile more (est = 1.1, 95% CI = [0.01; 2.2]). However, at 16 weeks old, that difference was gone.

The hybrid influenced the chickens' movement during both the VATs: at both ages, ISA Brown chickens walked straight towards the experimenter more often compared to Dekalb White chickens, which walked around more (VAT1: est = −1.8, 95% CI = [−3.1; −0.6]; VAT2: est = −4.3, 95% CI = [−5.9; −3.1]) or remained immobile more (VAT1: est = −2.2, 95% CI = [−3.5; −1.2]; VAT2: est = −3.4, 95% CI = [−4.8; −2.2]). At 16 weeks of age, ISA Brown chickens also walked around more often than Dekalb White chickens, which remained immobile more (est = −0.9, 95% CI = [−1.7; −0.3]).

There was a round effect on the VAT at both ages: at 6 weeks old, chickens from round 2 walked around more often, while chickens from round 1 walked straight to the experimenter more (est = −41.5, 95% CI = [−174.8; −10.9]) or remained immobile more (est = 772.8, 95% CI = [36.3; 2553.4]). At 16 weeks old, chickens from round 1 remained immobile more, while chickens from round 2 walked around (est = 0.9, 95% CI = [0.2; 1.7]) or walked straight to the experimenter more (est = 1.1, 95% CI = [0.2; 2.0]).

There was an experimenter effect on the movement in the VAT at both ages ($p < 0.05$).

#### 3.1.2. Latency to approach

At 6 weeks old, it took the Dekalb White chickens 72 s longer to approach the experimenter compared to the ISA Brown chickens (95% CI = [55.3; 89.5], $p < 0.001$). There was no effect of the incubation treatment (est = −6.8, 95% CI = [−23.88; 10.32], $p = 0.42$), the interaction between hybrid and incubation (est = −24.2, 95% CI = [−59.44; 11.13], $p = 0.18$), or the round (est = 18.5, 95% CI = [−11.8; 48.7], $p = 0.23$) on the latency to approach. There was also no experimenter effect ($p > 0.39$).

At 16 weeks old, it took the Dekalb White chickens 71 s longer to approach the experimenter compared to the ISA Brown chickens (est = 71.1, 95% CI = [47.39; 94.74], $p < 0.001$) (Fig. 2). There was no effect of the incubation treatment (est = 5.5, 95% CI = [−18.4; 29.4], $p = 0.65$), the interaction between hybrid and incubation (est = −3.5, 95% CI = [−51.4; 44.4], $p = 0.89$), or the round (est = −2.3, 95% CI = [−34.5; 29.8], $p = 0.89$) on the latency to approach. There was an experimenter effect on the latency to approach ($p \leq 0.034$).

#### 3.1.3. Latency to peck

At 6 weeks old, it took the Dekalb White chickens 81 more seconds to peck compared to the ISA Brown chickens (95% CI = [55.3; 89.5], $p < 0.001$). The chickens incubated with the light-dark cycle tended to take less time (22 s less) to peck compared to the chickens incubated in the dark (95% CI = [−23.88; 10.32], $p = 0.07$). There was no effect of the interaction between hybrid and incubation (est = −23.0, 95% CI =

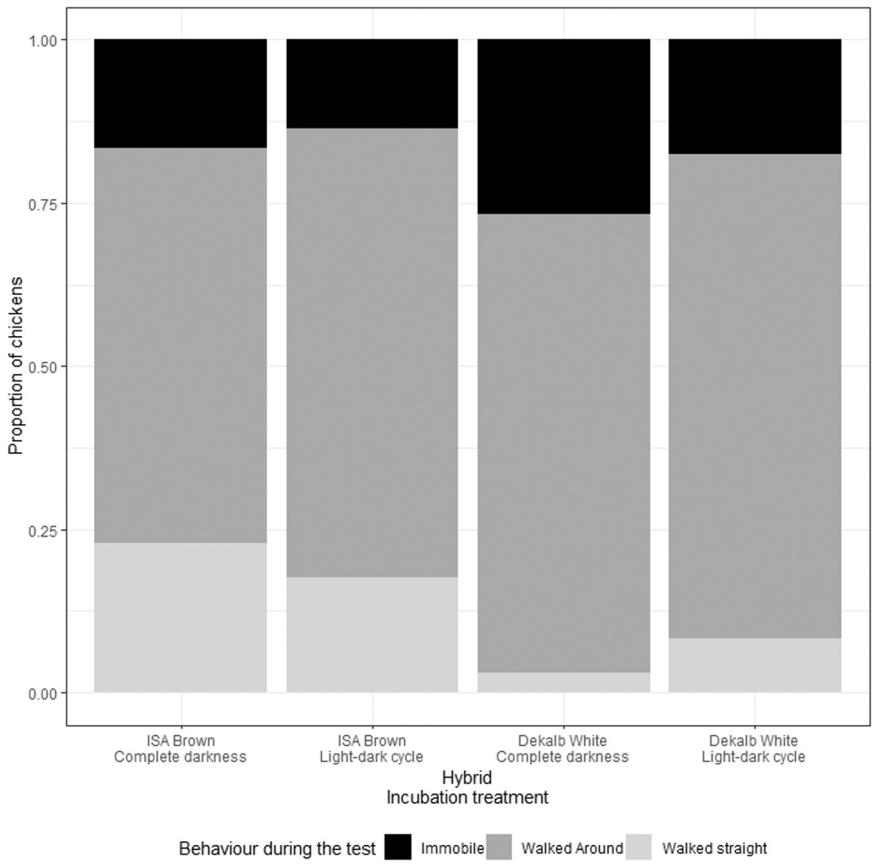


**Fig. 1.** Proportion of chickens that performed each movement during the VAT1 depending on the hybrid and incubation treatment. A gradient of color was used to describe the behaviors from the most fearful (immobile) to the least fearful (walked straight). The results are given in proportions as the sample sizes of each treatment group were not exactly the same.

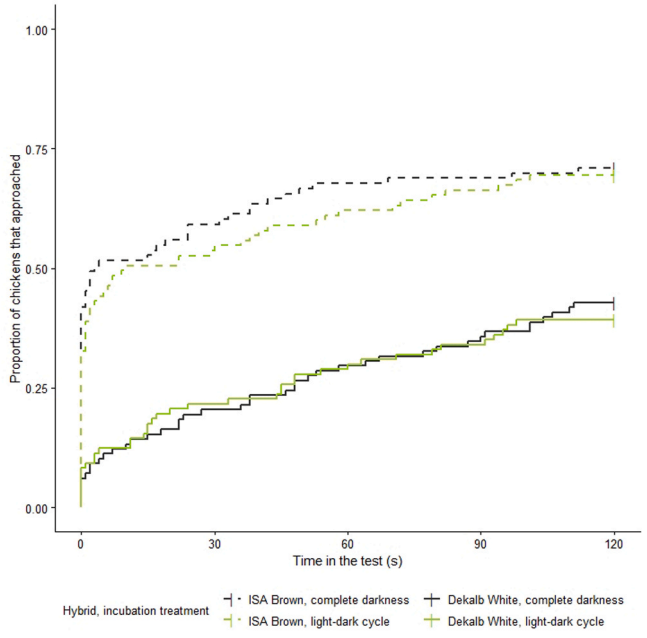


**Fig. 2.** Latency to approach the experimenter during the VAT2 depending on the hybrid and incubation treatment.

[−29.8; 54.8], $p = 0.38$) or of the round on the latency to peck at 6 weeks old (est = 18.2, 95% CI = [−11.8; 48.7], $p = 0.64$). There was also no experimenter effect ($p > 0.11$).

At 16 weeks old, it took Dekalb White chickens 108 s longer to peck compared to the ISA Brown chickens (95% CI = [89.3; 127.6], $p < 0.001$). There was no effect of the incubation treatment (est = −11.9, 95% CI = [−37.5; 13.7], $p = 0.36$), the interaction between hybrid and incubation (est = −1.6, 95% CI = [−45.2; 42.1], $p = 0.94$) or the round (est = −19.5, 95% CI = [−82.9; 43.9], $p = 0.55$) on the latency to peck. There was an experimenter effect on the latency to peck ($p \leq 0.049$).

### 3.2. Human approach test

#### 3.2.1. Latency to approach

The light-incubated chickens tended to approach the experimenter faster than the dark-incubated chickens during the HA test (est = −6.2, 95% CI = [−12.8; 0.4], $p = 0.066$). The latency to approach the experimenter was not influenced by the hybrid (est = −0.4, 95% CI = [−7.1; 6.3], $p = 0.91$), the interaction between incubation and hybrid (est = 7.7, 95% CI = [−5.6; 21.1], $p = 0.26$) or the round (est = 1, 95% CI = [−5.9; 7.8], $p = 0.78$).

#### 3.2.2. Latency to touch

The latency to touch the familiar experimenter was not influenced by the hybrid (est = 16.2, 95% CI = [−54.8; 87.3], $p = 0.65$), the incubation treatment (est = −38.9, 95% CI = [−109.7; 31.9], $p = 0.28$) or the interaction between these two (est = −62.7, 95% CI = [−204.2; 78.8], $p = 0.39$). The chickens in round 2 touched the experimenter 113.5 s later than the chickens in round 1 (95% CI = [43.2; 183.8], $p = 0.002$).

### 3.2.3. Reaction to capture simulation

The reaction to the CS was not influenced by the incubation treatment ($p > 0.53$). More Dekalb White chickens fled compared to ISA Brown chickens (est = 0.83, 95% CI = [0.4; 1.3], $p < 0.001$), but the other reactions were not influenced by the hybrid ($p > 0.53$). The interaction between incubation and hybrid did not influence the reaction to the CS ($p > 0.6$). More chickens approached during round 2 than round 1 (est = 1.39, 95% CI = [0.002; 3.3], $p = 0.08$), but the other reactions were not influence by the round ($p > 0.55$).

## 3.3. Tonic immobility test

The Dekalb White chickens had a shorter TI duration of 47 s compared to the ISA Brown chickens (95% CI = [−67.3; −27.3], $p < 0.001$) (Fig. 3). There was no effect of the incubation treatment (est = 0.2, 95% CI = [−30.6; 7.1], $p = 0.33$), the interaction between hybrid and incubation (est = 7.7, 95% CI = [−35.0; 50.4], $p = 0.72$), or the round (est = 43.5, 95% CI = [−124.154; 211.137], $p = 0.61$). There was an experimenter effect ($p \leq 0.031$).

There was no effect of the hybrid ($p \geq 0.18$), the incubation treatment ($p \geq 0.76$), the interaction between them ($p \geq 0.51$) or the round ($p \geq 0.43$) on the number of inductions.

## 3.4. Manual restraint test

### 3.4.1. Latency to struggle

During the manual restraint test, it took the Dekalb White chickens 137 s longer to start struggling than the ISA Brown chickens (95% CI = [108.6; 164.8], $p < 0.001$). There was no effect of the incubation treatment (est = −10.0, 95% CI = [−39.6; 19.5], $p = 0.51$), of the interaction between hybrid and incubation (est = −7.6, 95% CI = [−67.7; 52.5], $p = 0.80$) or of the round (est = 40.2, 95% CI = [−32.4; 111.9], $p = 0.27$) on the latency to struggle. There was an experimenter effect ($p \leq 0.049$).

### 3.4.2. Struggle rate

During the manual restraint test, the Dekalb White chickens performed fewer struggles than the ISA Brown chickens (est = −1.2, 95% CI = [−1.9; −0.5], $p = 0.002$). There was no effect of incubation (est = −0.03, 95% CI = [−0.7; 0.7], $p = 0.93$), of the interaction between hybrid and incubation (est = −0.069, 95% CI = [−1.45; 1.32], $p = 0.93$) or of the round (est = 1.0, 95% CI = [−0.8; 2.8], $p = 0.28$) on the struggle rate. There was an experimenter effect ($p \leq 0.025$).

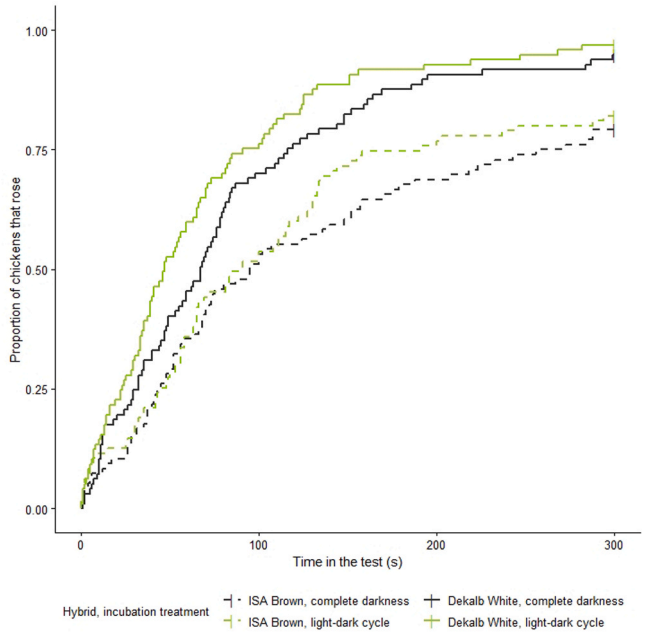


**Fig. 3.** Tonic immobility test duration depending on the hybrid and incubation treatment.

### 3.4.3. Latency to vocalize

It took the Dekalb White chickens 62 s longer than the ISA Brown chickens to start vocalizing (est = 61.88, 95% CI = [47.60; 76.16], $p < 0.001$) during the MR test. There was no effect of the incubation treatment (est = −0.17; 95% CI = [−10.5; 10.2], $p = 0.97$), the interaction between incubation and hybrid (est = −4.84, 95% CI = [−27.6; 17.9], $p = 0.68$) or of the round (est = −10.54; 95% CI = [−33.4; 12.4], $p = 0.37$) on the latency to vocalize. There was an experimenter effect ($p \leq 0.042$).

### 3.4.4. Vocalization rate

The Dekalb White chickens vocalized less than the ISA Brown chickens during the MR test (est = −7.93, 95% CI = [−10.12; −5.74], $p < 0.001$). There was no effect of the incubation treatment (est = −1.07, 95% CI = [−3.26; 1.12], $p = 0.36$) or of the interaction between incubation and hybrid (est = 0.146, 95% CI = [−4.23; 4.51], $p = 0.95$) on the vocalization rate. The chickens from round 2 tended to vocalize more than the chickens from round 1 (est = 5.50, 95% CI = [−0.48; 11.37], $p = 0.075$). There was an experimenter effect ($p \leq 0.049$).

### 3.4.5. Corticosterone levels in plasma

The baseline plasma corticosterone level was lower than the peak and recovery levels for all four treatments ($p < 0.001$) (Fig. 4). The peak level was higher than the recovery level for all groups ($p < 0.04$) except for the ISA Brown chickens incubated in complete darkness ($p = 0.28$).

The baseline plasma corticosterone level was 0.09 ng/mL lower in Dekalb White than in ISA Brown chickens (est = −0.09, 95% CI = [−0.12; −0.05], $p < 0.001$). There was no effect of the incubation treatment (est = −0.02, 95% CI = [−0.06; 0.02], $p = 0.28$), of the interaction between hybrid and incubation (est = 0.02, 95% CI = [−0.05; 0.10], $p = 0.59$), or of the round (est = −0.01, 95% CI = [−0.04; 0.03], $p = 0.61$) on baseline plasma corticosterone level.

The peak plasma corticosterone level was not affected by the hybrid (est = 0.07, 95% CI = [−0.09; 0.25], $p = 0.44$), the incubation treatment (est = −0.07, 95% CI = [−0.24; 0.10], $p = 0.43$), the interaction between hybrid and incubation (est = −0.08, 95% CI = [−0.43; 0.25], $p = 0.69$), or the round (est = −0.07, 95% CI = [−0.52; 0.44], $p = 0.77$). The experimenter had an effect on the peak corticosterone level ($p = 0.01$).

The recovery plasma corticosterone level was not affected by the hybrid (est = −0.08, 95% CI = [−0.24; 0.07], $p = 0.36$), the incubation treatment (est = 0.07, 95% CI = [−0.09; 0.22], $p = 0.43$), the interaction between hybrid and incubation (est = 0.19, 95% CI = [−0.10; 0.51], $p = 0.27$), or the round (est = −0.06, 95% CI = [−0.56; 0.37], $p = 0.81$). The experimenter had an effect on the recovery corticosterone level ($p = 0.02$).

## 4. Discussion

The aim of this study was to investigate the effect of lighted incubation in two laying hen hybrids on fear of humans.

The five tests performed aimed at investigating different aspects of fearfulness: with different human-chicken interactions, in a group (HA) or individually (VAT, TI, MR), at different ages (VAT1 and 2), and behavioral (all) or physiological (MR) responses. Despite the differences between the tests, clear patterns emerged from this investigation (Fig. 5): light during incubation only decreased fearfulness for one measurement (movement during the VAT1), and had no effect on the others. The Dekalb White chickens were more fearful than the ISA Brown chickens for 11 of the 15 behavior measurements, though one (TI duration) showed the opposite, and 3 showed no difference between

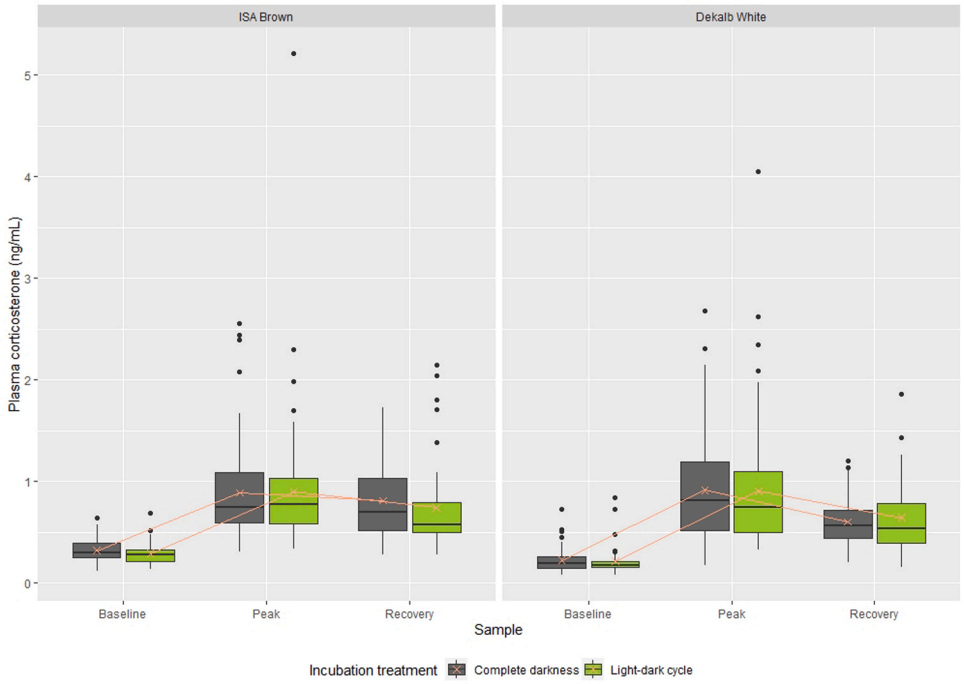


**Fig. 4.** Plasma corticosterone concentrations before (baseline), 15 (peak) and 30 min (baseline) after the MR test, depending on the hybrid and the incubation treatment. The boxplots show the median (bold line), first and third interquartile (bottom and top of the box), 95% range of the data (whiskers) and outliers (first and last 2.5% of the data range, dots). The beige crosses represent the averages, and the beige lines the average linear change in corticosterone between samples.

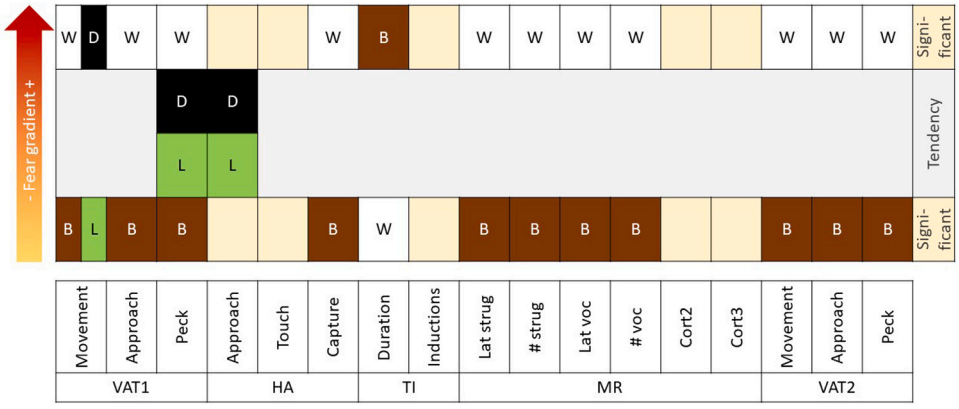


**Fig. 5.** Summary of the significant results and tendencies against a fear gradient described for each test parameter in the methods. The fear gradient has no unit and is relative to each individual factor and individual test parameter. As a result, white (W) and brown (B) chickens are compared to each other within each test parameter; light-incubated (L) and dark-incubated (D) chickens are compared to each other within each test parameter. For example, the movement in VAT1 shows that white chickens were more fearful than brown chickens and that dark-incubated chickens were more fearful than light-incubated chickens; in VAT2, only the hybrid difference remained; however, this does not give information on whether the white chickens experienced the same level of fear in VAT1 than in VAT2. When a factor is absent from a given parameter, it means it did not influence said parameter. E.g.: there was no significant difference in TI duration between the incubation treatments. All interactions were non-significant and therefore left out for this figure. Finally, though physiological stress and fearfulness are two different traits, they are presented here on the same gradient for easy reading. Parameters: Approach: latency to approach. Peck: latency to peck. Capture: reaction to capture simulation. Lat strug: latency to struggle. # strug: number of struggles per minute. Lat voc: latency to vocalise. # voc: number of vocalisations per minute. Cort2: change in corticosterone level between baseline and peak. Cort3: change in corticosterone level between recovery and peak. Tests: VAT1: voluntary approach test at young age. HA: human approach test. TI: tonic immobility test. MR: manual restraint test. VAT2: voluntary approach test at older age.

hybrids (latencies to approach and to touch in the HA test, and the number of inductions in the TI test). No effect of the incubation or of the hybrid was found on corticosterone responses to the MR. Finally, no significant interactions between incubation and hybrid were found for any of the parameters measured.

The chickens incubated with the light-dark cycle were less fearful than the chickens incubated in complete darkness for 1 of the 15 measurements (movement in the VAT1). It is worth noting, though, that 2 other measurements (latency to peck in the VAT1 and latency to approach in the HA) showed a tendency of the light-incubated chickens to perform less fearful behaviors than dark-incubated chickens. The direction of the difference is consistent with our expectations and literature, as light during incubation decreased fearfulness and stress sensitivity in several studies on chickens (Archer and Mench, 2017, 2014b, 2014a, 2013; Özkan et al., 2022, 2012b, 2012a). This significant result and these tendencies were present in the earliest tests performed (5 and 10 weeks of age). Broilers typically live 6–8 weeks, and, in literature, the effect of light during incubation on fearfulness in broilers is therefore always tested in the first 8 weeks of life. This corresponds to the timeframe in which we found significant results and tendencies. However, the laying hens in this study were tested until 17 weeks of age, which may explain the overall weaker impact of light during incubation

on laying hens compared to broilers.

Since more light goes through Dekalb White than through ISA Brown eggshell (Manet et al., 2023), the absence of significant interaction effect between incubation and hybrid is surprising. Indeed, we expected at least an effect on the Dekalb White chickens. It is possible that a significant interaction would emerge in other types of observations, such as exploratory behavior or cognition.

The Dekalb White chickens showed more fearfulness than the ISA brown chickens most of the time, which is consistent with our expectations and literature on other white and brown layer hybrids (*e.g.* Nelson et al., 2020; Uitdehaag et al., 2011, 2008a). To our knowledge, there are no clear explanations regarding the mechanism of the link between feather color and fearfulness towards humans. One could theorize the genes coding for each of those traits are co-dependent.

The tests performed on the individual level (VAT1–2, TI, MR) resulted in Dekalb White chickens displaying more fearful behavior than ISA Brown chickens for all the parameters, though one exception is worth mentioning. In the TI test, the Dekalb White chickens rose sooner, and therefore showed less fearfulness, than the ISA brown chickens. Most studies performing a TI test on white and brown laying hens found opposite results, with brown chickens rising sooner (*e.g.* Albentosa et al., 2003; Fraisse and Cockrem, 2006; Jones and Faure, 1981a; Pusch et al., 2017). However, a few studies found similar results to ours (Campo et al., 2006; Peixoto et al., 2020). Especially, (Jones and Faure, 1981b) found that in chickens used to being handled, but not in control chickens, white laying hens had a shorter TI duration than brown laying hens. Considering the chickens in this research underwent 4 behavior tests and 6 weighing moments requiring handling before the TI test, they were likely used to being handled on the day of this test.

Tonic immobility being an anti-predator strategy, it is the least human-related test of this paper. Indeed, tonic immobility was induced by the experimenter, rather than provoked by them. This could explain the different pattern found in TI duration. This implies, however, that hybrid differences in fearfulness are context-dependent. As a result, investigations should not solely rely on the TI test, and especially not in production animals, where human-animal relationships are important. Instead, a (combination of) context-relevant test(s) would give more meaningful information.

In the HA test, performed on group level, no hybrid difference was found in terms of latency to approach or to touch the experimenter. In literature, differences were found for this test, with white laying hens being less (Jones and Faure, 1981b) or more (De Haas et al., 2014a, 2013; Jones and Faure, 1981b; Odén et al., 2002) fearful of humans than brown laying hens. It is possible that the chickens in this research felt safer in their home pen and with their peers than during the other tests, for which they always were taken to a different, less familiar room and were socially isolated. The latter testing conditions may have increased hybrid differences in fear levels, masked or absent in a group context.

In addition, the HA test is unique as, at the beginning, the experimenter did not intend any interaction with the chickens, contrary to the VAT, TI and MR tests. In this context, chickens may have been less fearful, making it more difficult to find differences in fearfulness. Indeed, the CS (the last part of the HA, during which the experimenter intended an interaction with the chickens) showed hybrid differences, supporting this hypothesis. Overall, the results of the HA test highlight that fear of humans is likely context-dependent in laying hens.

The most surprising results are those of the MR test: based on the behavior, Dekalb White chickens appeared more fearful than ISA Brown chickens. However, corticosterone levels did not significantly differ between both hybrids. These results suggest that the behavior expressed during the test shows the difference in stress coping strategies of the chickens rather than the difference in fearfulness.

Some previous studies found that brown laying hens were more active than white ones in an MR test (Uitdehaag et al., 2011, 2008b), but one found that they had similar behavior (Uitdehaag et al., 2008b); regarding the corticosterone levels, most studies found no difference between white and brown laying hens (Brown et al., 2022; Rozempolska-Rucińska et al., 2020; Uitdehaag et al., 2011, 2008b), though some found higher corticosterone levels in white laying hens (Fraisse and Cockrem, 2006; Peixoto et al., 2021; Uitdehaag et al., 2008b) and others in brown laying hens (Brown et al., 2022).

Multiple studies have performed the MR test on chickens, and results are indeed not consistent in terms of the relation between behavior and corticosterone: some found that more active chickens had lower corticosterone levels than less active chickens (Cockrem, 2007; Jones and Hocking, 1999); others found that more active chickens had similar corticosterone levels to less active chickens (Bolhuis et al., 2009; Campbell et al., 2016; Uitdehaag et al., 2008b; van der Eijk et al., 2019); yet others found that chickens with high corticosterone had similar behavior response to chickens with low corticosterone (Rodenburg et al., 2009; Uitdehaag et al., 2008b). This relationship differed sometimes within the same study depending on age, line, or bird condition (*e.g.* beak-trimmed or not), regardless of their plumage color (Brown et al., 2022; Uitdehaag et al., 2008b). The relationship between corticosterone and behavior therefore deserves further investigation. Non-domesticated animals usually display similar behavioral and hormonal profiles, but this seems to be different in laying hens, as suggested recently (Peixoto et al., 2021).

This research was performed to improve our understanding of laying hen welfare, and the roles hybrid and light during incubation play in it. Here, we define welfare as a dynamic concept including both physical and affective states: "an individual animal is likely in a positive welfare state when it is mentally and physically capable and possesses the ability and opportunity to react adequately to sporadic or lasting appetitive and adverse internal and external stimuli, events, and conditions" (Arndt et al., 2022; Ohl and van der Staay, 2012).

It is important to note that fear responses are an adaptive part of the behavioral repertoire of an animal. Therefore, decreased fearfulness does not necessarily mean improved welfare. As shown by the results of the MR test, animals that hardly show fearful behavior may still mount a pronounced physiological stress response. The selection on less fearful animals should focus on the animal, and its capability to reach a positive mental state, rather than improving practical conditions such as handling of the animals. Coping abilities may be supported by offering appropriate environmental conditions, starting prenatally.

In conclusion, light during incubation decreased fearfulness in the earliest test performed, but not in the others. Hybrid differences were found, where Dekalb White chickens were more fearful for most measurements, though sometimes equally or less, than ISA Brown chickens.

The clear differences between Dekalb White and ISA Brown chickens call for hybrid-specific production guidelines and research: the environment and management procedures that allow a certain hybrid to cope with production animal life (and therefore have a better welfare) can be different than that of another hybrid. Similarly, research outcomes found on a specific hybrid do not necessarily apply to all laying hen hybrids (De Haas et al., 2014b).

Future research should focus on other aspects of fearfulness and stress in different laying hen hybrids. The effect of light during incubation on laying hens should also be investigated in other contexts. Namely, other fearfulness contexts (*e.g.* novelty or social isolation) would be interesting to look into, as the results are not consistent in this paper nor in literature (Albentosa et al., 2010; Nelson et al., 2020; Pusch et al., 2017). In addition, light during incubation can affect physiology and brain development (Rogers, 1982; Saito et al., 2005) and cognitive capacities (Daisley et al., 2010). It would therefore also be pertinent for HPA-axis activity, histology and cognition investigations to take place.

## Ethical statement

The research project was approved by the central authority for scientific procedures on animals (Centrale Commissie Dierproeven (CCD), the Hague, the Netherlands) under the number AVD1080020198685.

All procedures were performed by a trained person, certified or under the supervision of a certified person.

## CRediT authorship contribution statement

**MWE Manet:** conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article. **S Kliphuis:** conception and design of the study, acquisition of data, analysis and interpretation of data, revising it critically for important intellectual content. **RE Nordquist:** conception and design of the study, analysis and interpretation of data, revising it critically for important intellectual content. **VC Goerlich:** conception and design of the study, analysis and interpretation of data, revising it critically for important intellectual content. **FAM Tuyttens:** analysis and interpretation of data, revising it critically for important intellectual content. **TB Rodenburg:** conception and design of the study, analysis and interpretation of data, revising it critically for important intellectual content.

## 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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