Animal Behavior and Cognition
ABC 2025, 12(1):45-68
DOI: https://doi.org/10.26451/abc.12.01.03.2025

Shining Light On The Laying Hen Brain: The Effect Of Light During Incubation Depends On Cognitive Task And Hybrid

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Citation – Manet, M. W. E., Kliphuis, S., Bas Rodenburg, T., Goerlich, V. C., Tuyttens, F. A. M., & Nordquist, R. E. (2025). Shining light on the laying hen brain: the effect of light during incubation depends on cognitive task and hybrid. *Animal Behavior and Cognition, 12*(1), 45-68. https://doi.org/10.26451/abc.09.01.03.2022

Abstract – The influence of early life on animal welfare later in life is increasingly recognized as important. In chickens, a promising early-life intervention is the exposure to light during incubation. Due to the position of the embryo in the egg, the two brain hemispheres receive different amounts of light stimulation via the eyes. This unequal stimulation results in increased lateralization of the brain, which is the specialization of brain hemispheres on certain functions. In current commercial practice, chickens are incubated in complete darkness. Information about the effect of light during incubation on laying hen cognition is scarce, although stronger effects are expected in hybrids coming from white eggs than brown eggs. In this experiment, we therefore incubated eggs of ISA Brown (ISAb) and Dekalb White (DW) layer hybrids (N = 1200) either in a green light-dark cycle (light), or in complete darkness (dark). At hatching, brains were collected from 80 individuals. Tyrosine hydroxylase and DoubleCortin levels were investigated, but no significant differences were found between incubation treatments or hybrids. Then, cognition tests were performed on the surviving females. In a detour test (N = 198), light-exposed chicks showed 24% more lateralized behavior (i.e., turned left from the obstacle) than chicks incubated in the dark, regardless of the hybrid. In a 5-day holeboard test (N = 78), adult hen performance results were inconsistent. Finally, during a social recognition test (N = 76), ISAb-dark showed significantly greater interest for the familiar hen, whereas DW-light showed greater interest for the unfamiliar hen. To conclude, light during incubation affected the brains and cognition of laying hens at different life stages post-hatching, although the effects were not consistent across tests or hybrids.

Keywords – Chicken welfare, Prenatal, Neurobiology, Cognition, Lateralization

Chickens have demonstrated strong cognitive capacities; for example, recognizing partially or completely occluded objects, performing arithmetic operations and perceiving time intervals (for a review, see Marino, 2017). To provide adequate welfare, it is therefore important that domesticated chicks are sufficiently cognitively stimulated (Ferreira et al., 2021; Nordquist, 2021). To ensure sufficient stimulation, increasing knowledge on chicken cognition needs is required. We aimed to investigate how current industry practices (namely, dark incubation) affect cognitive capacities in chickens, by comparing them to practices that better resemble the chickens' natural environment.

Commercial poultry eggs are typically incubated in constant darkness. One popular intervention in research is to assess the presence of light during incubation, to mimic the hen leaving the nest in nature

(Archer & Mench, 2014a). Light during incubation improved broiler chick welfare by decreasing fearfulness in several behavior tests (Archer & Mench, 2017). In laying hen chicks (from here on, layer chicks), however, light during incubation decreased fearfulness in only one of five human fear tests (Manet, Kliphuis, Nordquist, et al., 2023). Light during incubation is especially interesting to investigate in laying hens, as their production cycle is longer than broilers', meaning that layer hens live to adulthood whereas broilers are slaughtered as chicks. This makes it relevant to explore effects of light exposure during incubation on laying hen welfare in later life.

The mechanisms of the effects of light during incubation on chickens make cognitive capacities particularly interesting to explore. Indeed, on embryonic day (ED) 17, the embryo turns in the egg and exposes its right eye towards the shell, whereas the left eye is hidden by the body. The two brain hemispheres are then unequally exposed to light for the last days of incubation. That results in increased brain lateralization compared to dark incubation: the brain is anatomically asymmetrical, and each hemisphere specializes into specific functions, improving the individual's performance (e.g., Rogers, 1982; Vallortigara & Rogers, 2020). Although anatomical asymmetry exists in dark-incubated chicks (e.g., c-Fos expression in the preoptic area, and spontaneous firing rate of visually responsive units in the visual Wulst), it is enhanced by light during incubation in some brain areas (e.g., c-Fos expression in the septum, and response to a contralateral eye stimulation in the visual Wulst; Costalunga et al., 2021; Lorenzi et al., 2019). Similarly, behavioral lateralization is also observed in dark-incubated chick; for example, in social pecking behavior (Vallortigara et al., 2001), although not in, for example, attack and copulation (Deng & Rogers, 2002; Zappia & Rogers, 1983). Specific tasks are better performed by light-incubated chicks. For example, visually-guided behaviors, such as foraging or scanning the air for predators, are better performed by chicks incubated with light compared to dark-incubated chicks (Rogers, 2012; Rogers et al., 2004). Layer chicks incubated with light are also better at transitive inference (Daisley et al., 2010), position discrimination (Chiandetti & Vallortigara, 2009) and imprinting (Andrew et al., 2004) than layer chicks incubated in darkness. By improving these skills, light during incubation was beneficial for the chicks' welfare. Other cognitive tests should be performed to further compare cognitive capacities in dark- and light-incubated laying hens.

Another not-yet-well documented topic is the effect of light during incubation on anatomical brain development in laying hens. Previous studies have shown that the visual pathways are anatomically impacted by light exposure during incubation, as they develop asymmetrically (Deng & Rogers, 2009; Rogers & Sink, 1988). This explains the effects of light during incubation on visually guided behavior and cognition mentioned previously. However, the effects of light exposure during incubation on neurobiological pathways related to stress and brain plasticity are still to be investigated. Stress regulation and brain plasticity play essential roles in positive welfare, making this an important topic.

Several protocols of light during incubation have been tested. In addition to the critical period of the last days of incubation from ED17 (see above), research has shown that light during the earliest days of incubation can affect chicken lateralization (Chiandetti et al., 2013, 2017; Chiandetti & Vallortigara, 2019). The wavelength of light during incubation also influences the level of lateralization: exposure to white or to alternating green-red light resulted in a high level of lateralization, exposure to green light resulted in a lower level of lateralization, and exposure to red light or to darkness failed to induce lateralization in the pebble-floor task (Rogers & Krebs, 1996). In laying hens, an incubation protocol using 16L:8D green LEDs throughout incubation was shown to decrease feather pecking, a severe welfare problem (Özkan et al., 2022), although several studies demonstrated that 12L:12D was the best light exposure schedule to improve welfare as demonstrated by reduced fearfulness, reduced physiological stress response and better establishment of daily activity (Archer & Mench, 2013, 2014b, 2017; Ferreira dos Santos, 2019). The effects of green 12L:12D throughout incubation on laying hen brain development and cognitive capacities were therefore investigated and are reported in this paper.

Laying hens hatch from either white or brown eggs. In a previous paper, we showed that there is a stronger transmission of light through white eggshell compared to brown eggshell (Manet et al., 2023). It therefore seems plausible that light during incubation could affect white-egg laying hens differently than brown-egg hens, and this should be explored further.

In the present paper, we assessed the effect of light during incubation on brain development and cognition of ISA Brown (ISAb) and Dekalb White (DW) hens, which were incubated either in complete darkness, or in a green light-dark cycle of 12L:12D throughout incubation. We first investigated tyrosine hydroxylase (TH) and doublecortin (DCX) levels in the brain of newly hatched chicks. TH is a rate-limiting enzyme involved in the production of dopamine (Nordquist et al., 2013), which itself plays an important role in positive welfare (Broom & Zanella, 2004). More specifically, dopamine is released in stressful situations (Hewlett et al., 2014), plays a crucial role in learning and memory, and is involved in reward processes and positive motivational affect (Tahamtani et al., 2016). DCX is expressed in migrating neurons and therefore highlights brain plasticity (Barnea & Pravosudov, 2011; Capes-Davis et al., 2005; Hannan et al., 1999; Piens et al., 2010), and it has been shown that neurogenesis continues after hatching in quails (Nkomozepi et al., 2019) and in chicks (Revilla et al., 1998). Brain plasticity is essential for captive animals to cope with stressors, and therefore plays a role in positive welfare (Arndt et al., 2022; Ohl & van der Staay, 2012). We therefore examined the potential of light during incubation to improve welfare as per TH and DCX expression. More specifically, we focused on TH and DCX levels in four areas of interest (AOIs): the medial striatum (MSt), the lateral striatum (LSt), the bed nucleus of stria terminalis (BST) and the amygdala. Indeed, the MSt and the LSt are known to play a role in visual functions (Marzluff et al., 2012; Melleu et al., 2016; Nkomozepi et al., 2019) and, therefore, have a high potential to be impacted by light during incubation (Johnston et al., 1997; Rogers & Deng, 1999). In addition, all four AOIs are involved in stress regulation and adaptive capacities (Bálint & Csillag, 2007; Crestani et al., 2013; Marzluff et al., 2012; Mayer et al., 2017; Medina et al., 2017; Melleu et al., 2016; Metwalli et al., 2023; Roth et al., 2012). Finally, the amygdala is also involved in social behavior (see *e.g.,* Mayer et al., 2019; Rosa-Salva et al., 2021), such as social recognition (Parada et al., 2021), a lateralized brain function (Rosa-Salva et al., 2012). Laying hens are a highly social species, and commercial housing conditions can be a challenging environment for chickens, requiring good adaptation capabilities. Investigating brain areas related to these capabilities is therefore highly relevant.

In addition, we examined the effect of light exposure during incubation on performance on three tests of cognition: a detour test, a holeboard test, and a social recognition test. The detour test, inspired by Vallortigara et al. (1999), was used to assess behavioral lateralization using a social stimulus at 3 or 4 weeks old, which is the age at which the same incubation protocol resulted in significant lateralization differences (Özkan et al., 2022). In a series of trials, the chicks could pass a barrier either from the left or from the right side to get to the social stimulus. The chosen side was used to calculate a lateralization index. The other two tests were performed at the beginning of the laying phase to investigate the long-term effects of light during incubation on laying hen cognition. The holeboard test, inspired by van der Staay et al. (2012), was used to evaluate spatial working memory at 21 weeks old. The hens had access to several rewarded cups. The number of times they went back to a cup they already ate the reward from, were counted as mistakes and used to calculate a memory index. Finally, the social recognition test, inspired by Hewlett and Nordquist (2019), was used to investigate whether laying hens could differentiate between a familiar and an unfamiliar hen of the same hybrid at 24 weeks old. The latency to visit each hen in a Y-maze, and the amount of time spent visiting them, were compared between incubation treatments and between hybrids to establish such capability. With these three cognitive tests, we aimed to uncover the effects of light during incubation on an increased range of cognitive capacities in laying hens.

Light-incubated chickens were expected to be more lateralized (Rogers, 1996; Vallortigara et al., 1999), reflected by stronger lateralization indexes, to have better welfare than dark-incubated chickens (Archer, 2017; Archer & Mench, 2013, 2014b, 2014a, 2017), reflected by higher levels of TH, and to have stronger cognitive skills (Daisley et al., 2010), reflected by higher DCX in the amygdala, a better working memory, and a better recognition of the familiar hen. DW hens were expected to show more interest in the unfamiliar hen compared to the ISAb hens in the social recognition test (Hewlett & Nordquist, 2019). Hybrids were not expected to differ in any other cognition test (Hewlett & Nordquist, 2019), although stronger effects of the light during incubation in DW were expected compared to ISAb, due to more light passing through DW eggshells than ISAb eggshells (Manet et al., 2023). As a result, the difference in

lateralization and welfare was expected to be larger between DW-light and DW-dark than between ISAb-light and ISAb-dark.

Method

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 in accordance with Dutch national law and the European directive 2010/63/EU. All procedures were performed by a trained person, certified with the article 9 of the Dutch Experiments on Animals Act or under the supervision of a certified person.

Animals

1200 fertilized eggs from ISA Brown and Dekalb White layers were provided by Hendrix Genetics. Parental flocks were between 37 and 50 weeks of age, and were housed in traditional single-tier housing at the hatchery Het Anker (Ochten, the Netherlands).

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 ED19, 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 (520nm) 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 L:D = 12:12 throughout incubation. The other two incubators were kept in complete darkness. The standard design was therefore a 2 x 2 factorial arrangement (hybrid x incubation treatment: ISAb-dark, ISAb-light, DW-dark, DW-light) repeated in two rounds.

At ED19, eggs were moved to hatching baskets. From ED19 to ED21, hatching and health quality checks were performed on new hatchlings every six hours. Hatching was checked with the room light turned on (Philips Master, TL-D, 36W/840). It took between a few seconds to two minutes each time, and the average light intensity at egg level was 534 lux for the HatchTech incubators (448 lux for the climate respiration chambers, measurements taken with the green light off). The health quality checks were performed with the same room light turned on, with, at chick level, at average light intensity of 560 lux for those from the HatchTech incubators (356 lux for the climate respiration chambers). Neck-tags with unique numbers were given to the female hatchlings for identification throughout the experiment. The hatchlings were then placed back in the hatching baskets. On ED20, which was the hatching peak, the brain collection occurred (see next section). On ED21, all hatched female chicks were transported in cardboard boxes from the WUR to their rearing facility at the Faculty of Veterinary Medicine of Utrecht University (UU) by a professional poultry transporter.

Brain Collection and Analysis

Although the focus of the study was on laying hens, such that males were killed upon hatching, we included the brains of male chicks in the analysis of brain lateralization to maximize the benefits of their inclusion up to this point in the study.

At hatching, the brains of 80 individuals (5 per treatment, per sex and per round) were collected to measure levels of Tyrosine Hydroxylase (TH) and of DoubleCortin (DCX). An experimenter collected a day-old chick from its incubator and took it to the dissection room next door, transporting it in a foam box. The experimenter then decapitated the chick with sharp scissors, and the brain was immediately dissected and immersed in 4% paraformaldehyde (PFA) (Klinipath, catnr. 4078.9010); brains were stored in PFA at 6°C for around two days, and then moved to 70% ethanol (Brunschwig Chemie, catnr. EA99-1718-10SD) and stored at 4°C until further use.

The detailed protocol of the rest of the lab work is available in supplementary materials (Protocol S1). Briefly, the brains were embedded in gelatin, and the brain was sliced in sections of 40 µm thickness using a vibratome (Leica, VT1200/S). Sections were incubated overnight in primary antibodies of either anti-TH (Merck Millipore, catnr. Ab152 lot nr. 3845256) or anti-DCX (Abcam catnr. Ab18723 lot nr. GR3414309), rinsed and incubated in biotinylated goat-anti-rabbit Igg (Vectastain catnr. PK-6101 lot nr. ZJ1205) for 1 hour at 37°C. Staining was visualized with 3,3'-Diaminobenzidine (Sigma-Aldrich, catnr. D4293) in 0.05M TBS with 0.02% H_2O_2, mounted on Superfrost slides, dehydrated through a series of ethanol and xylene, and coverslipped using DePeX (Serva Electrophoresis, Heidelberg).

Digital photographs from immunostained sections were taken on an Olympus microscope BX51 equipped with a UC50 digital camera. The AOI of the left and right side of each section was photographed with a magnification of 2x, a resolution of 2588 x 1960, an exposure time of 1,5 ms and with maximum light intensity and the diaphragm fully open. To measure the staining intensity of TH and DCX, ImageJ/FIJI (version 2.9.0) was used. Every area of interest was manually drawn as well as an empty area next to the section for the background intensity. The intensity of each AOI was subtracted from the intensity of the background. For each AOI and each staining, two parameters were measured:

a) The intensity lateralization: intensity in the right hemisphere - intensity in the left hemisphere

b) The total intensity: intensity in the right hemisphere + intensity in the left hemisphere

For the intensity lateralization, a positive number would mean that the intensity is higher in the right hemisphere than in the left; a negative number would mean that the intensity is higher in the left hemisphere than in the right.

Husbandry

Groups of 10 chickens from the same hybrid and same incubation condition were housed in 20 pens that were 246 x 88 cm, 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 lights (GlassLux Standard 1x36W Philips IP67 colour 830, Boon Agro) were used. In addition, the chicks 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 randomly distributed over the 20 pens, taking into account the location of the ceiling windows.

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

Until the chicks were 5 weeks old, the rearing environment was enriched with commercial dark brooders of 25 x 25 cm (WP-25, Comfort Chicks, OLBA B.V., Coevorden, the Netherlands). Dark brooders are used to mimic the presence of a mother by offering a warm and dark shelter. Their height was adjusted as the chicks grew. Black cotton fabric was cut and placed around the dark brooders as curtains with multiple openings to make the place even darker, and removed after one week. Wooden perches were also provided on the floor until 6 weeks old, thereafter replaced by a higher (61 cm high) wooden platform (61 x 88 cm) 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., 2014).

Welfare of the chicks was monitored on a daily basis: a handful of grains was provided in each pen every morning to easily spot weak or unresponsive 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, Newcastle Disease vaccines (Nobilis® ND Clone 30, MSD) were given on days 14, 68 and 95 by the spraying method.

Notable changes brought to the chicks 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 2) of leg bands with RFID tags at different stages of life for another research project. Finally, in round 1, three DW males were identified and removed at 5 weeks of age from three different pens.

At 18 weeks of age, most laying hens were removed from the experiment, keeping only one pen per treatment for the last two cognition tests in each round (the laying phase subset). The four groups were moved to the first four pens of the stable for practical reasons.

Cognition Tests

In total, three cognition tests were performed on the chickens: a detour test (3-4 weeks old), a holeboard test (21 weeks old) and a social recognition test (24 weeks old). They occurred 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 lights as in the home-pens were present in the testing rooms. Several experimenters performed the tests over the days and rounds. Strict definitions of each behavior were written beforehand and are available on the detailed protocols in the data repository. Each test started with a training to make sure experimenters scored the same. The chickens also underwent fear tests (Table S1). Some are reported in Manet et al. (2023). Only females were presented with the cognition tests. Indeed, including males would have required increased means and time, and by that reduced the quality of the investigation on females, which are the priority sex when it comes to laying hen research.

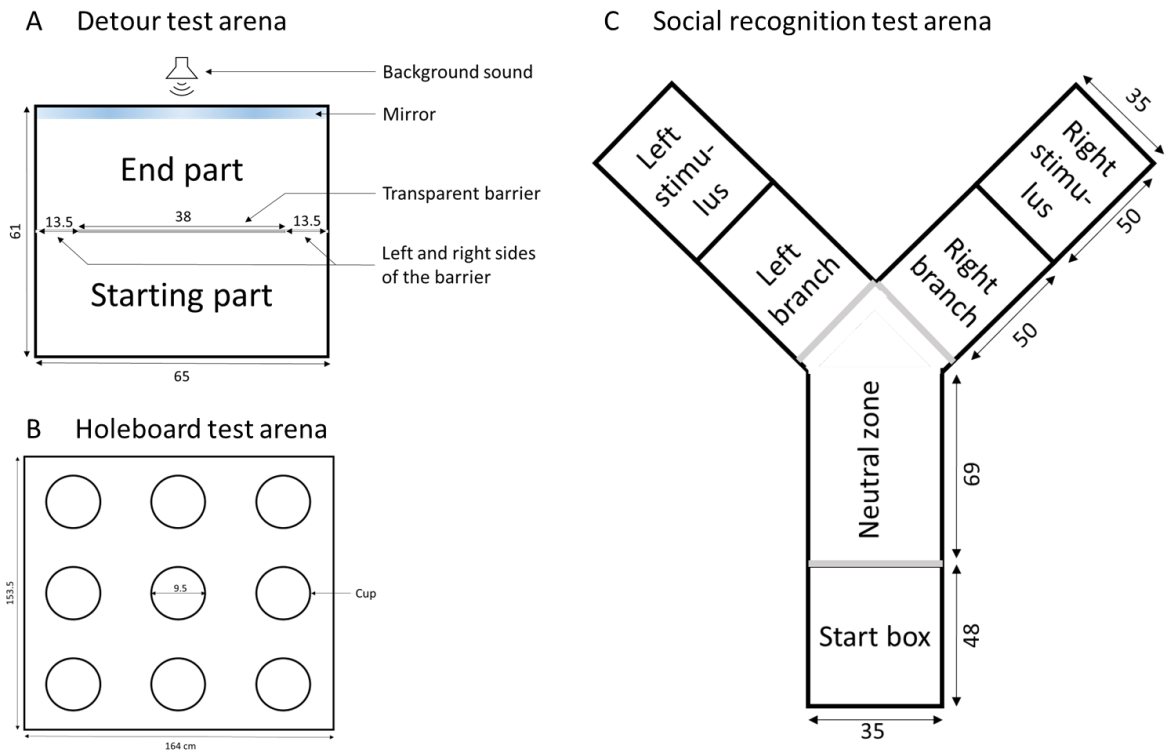
Detour Test

With means of an emergence test at 3 weeks of age, (S. Ozkan, personal communication, December 24, 2019) found that an 18L:6D cycle of green light induced stronger lateralization than incubation in darkness. Based on that, the 198 chickens from round 2 were individually tested in a detour test at 3-4 weeks of age. Each of them was tested six times in a row (six trials) before being taken back to its home pen.

The detour test took place in two test rooms in parallel, so that two chickens could be tested at the same time without influencing each other. The wooden test arenas were separated in two parts (starting and end parts) by a transparent barrier made of plastic (Figure 1A). The chickens could easily access the end part via either side of the barrier. The end part had a mirror parallel to the transparent barrier covering the whole wall to encourage social motivation. Behind the mirror, a recording of the stables with the home-pens was playing as a background sound, to decrease fearfulness and encourage social motivation. A camera was attached at a height of 161.5 cm (172.5 in the other room) for the experimenters to observe the chickens on a monitor while staying out of their sight.

Figure 1

Top View of the Test Arenas



Note. Numbers are dimensions in cm. A: detour test arena (height = 61 cm). B: Holeboard test arena (arena height = 100 cm; cup height = 5 cm). C: Social recognition test arena (height = 45.5 cm).

Chicks were caught and brought individually to the test room. The chicks were placed in the middle of the starting part, facing the transparent barrier and the mirror. A grid was placed on top of the arena to prevent escapes, and the test began. The chicks were given a maximum of 5 minutes to pass the barrier and access the end part. A chick had passed the barrier when its full head and neck were in the end part. If the chick passed the barrier, the side of the detour was scored (left or right). If the chick had not passed the barrier, it was gently caught by hands and placed in the end point from above the barrier. The chicks were given around 10 seconds in front of the mirror as a reinforcement before being caught again for the next trial. There were six trials in a row per chick, to get a maximum of six detours to one side or the other. Throughout the procedure, particular attention was paid to keep the set-up and the chick handling as symmetrical as possible, to avoid influencing the chicks' lateralization.

A lateralization index (LI) was then calculated:

$$(R-L)/(R+L)*100,$$

where R = number of detours to the right, and L = number of detours to the left (Vallortigara et al., 1999).

Chicks that never passed the barrier were excluded from the analysis as no LI could be calculated. Chicks that passed the barrier in only one of the trials were also excluded, because repeatability is essential to assess lateralization in a detour test (Vallortigara et al., 1999). An analysis was also conducted on the data from the chicks that passed the barrier in the six trials, excluding all others, and the results were similar (data not shown).

In round 1, 38% of the chicks made 0 or 1 choice, and only 38% made 6 choices (Table 1). Because of the high fear component, a habituation to the arena was performed on the chicks from round 2. The chicks were placed at the start point with all their penmates for two minutes, then again with half their penmates for one minute, then in pairs for one minute, and one last time alone for one minute. That habituation led to 0.5% of the chicks making 0 or 1 choice, and 95% to make 6. The habituation clearly having an effect on the chicks' behavior during the test, the chicks from round 1 (and the high fear component) were excluded from the analysis.

Table 1

Percentage Of Chicks Per Number Of Detours Made During The Detour Test In Rounds 1 And 2

	Number of detours made						
Round	0	1	2	3	4	5	6
1	31.75	5.82	2.65	6.88	6.35	8.47	38.10
2	0.51	0.00	0.51	1.52	0.51	2.02	94.95

Holeboard Test

We have previously demonstrated that layer chicks show high levels of working memory already at early ages (7-9 weeks of age: Nordquist et al. (2011); 9-11 weeks of ages: Hewlett & Nordquist (2019)). Tahamtani et al. (2015) furthermore showed that laying hens are capable of performing a holeboard test at the beginning of the laying phase (18-23 weeks old).

To look into the long-term effects of light during incubation on laying hen cognition, a holeboard test was performed on 78 hens (1 pen per treatment and per round, randomly selected) at 21 weeks old. The week before, the hens were habituated to red plastic cups and re-introduced to a food reward used in previous tests (Manet, Kliphuis, Nordquist, et al., 2023): dry mealworms. The habituation took place in the home-pens, with the experimenters outside the home-pens, although still visible from the hens. The habituation was considered successful when at least two hens from each pen had pecked at the red cup (or the mealworms in them). Because the hens readily ate the mealworms, it was deemed unnecessary to deprive them of food prior to the test.

The holeboard test followed the protocol from Hewlett and Nordquist (2019). It took place in a wooden arena (Figure 1B) with a camera placed at 202 cm of height for the experimenters to observe the hens while staying out of their sight. Nine red cups of 200 mL (10 x 10 x 5 cm) were evenly spaced on the floor of this arena, with a piece of dry mealworm in each cup. The hens were tested individually, once a day, five days in a row each (except for one hen that skipped the fourth day to recover from an injury caused by penmates). The number of different cups visited (maximum 9), the total number of cups visited (including revisits), the latency to revisit a cup and the latency to finish the test were scored. A hen had visited a cup when it had pecked at the food reward in it; it had revisited a cup when pecking for the second time in a cup; and it had finished the test either when all nine cups had been visited, or after five minutes – whichever came first.

From then, a working memory index was calculated: number of different cups visited / total number of cups visited. The score could range from 0 to 1, and a low working memory index was associated with poor performance (van der Staay et al., 2012). The hens that visited less than 7 different cups were excluded from the analyses of this parameter. A short latency to revisit was associated with poor performance, and a long latency to finish the test could be explained by poor performance or low motivation (van der Staay et al., 2012).

The trials were analyzed separately: learning and habituation-sensitization effects through repetition were possible and could have influenced the results if the data were pooled (Blumstein, 2016). Light-incubated hens were expected to show better learning than dark-incubated hens (Chiandetti & Vallortigara, 2009), demonstrated by a steeper learning curve. No hybrid difference was expected in terms

of learning (Dudde et al., 2018; Hewlett & Nordquist, 2019). There are no published studies on habituation-desensitization in chicken behavior, so we did not formulate any expectation in this regard.

Social Recognition Test

Adult hens are capable of social recognition (Abeyesinghe et al., 2009; Bradshaw, 1991; D'Eath & Keeling, 2003). To investigate whether light during incubation could affect that ability, a social recognition test was performed on 76 hens (the same individuals as for the holeboard test, minus two hens that had reached a humane end point) at 24 weeks old. Each hen was tested once a day for 5 days in a row, except for 10 hens in round 1 that were tested only 4 days (with a maximum of one day without testing) for practical reasons, and one hen in round 2 that was tested only 2 days (in a row), after which it had to be socially isolated because of injuries due to penmates. The test took place in a wooden Y-maze (Figure 1C) with a camera placed at 205 cm of height for the experimenters to observe the hens while staying out of their sight. The maze was covered with wire mesh to avoid any escape.

The test hen was placed in the start box, while two stimuli hens were placed in the stimuli boxes at the end of each branch of the Y-maze. The two stimuli hens were of the same hybrid as the test hen; one was from the same home-pen (*i.e.*, familiar hen), and the other from a different pen (i.e., unfamiliar hen). The experimenters were blind to which stimulus hen was the familiar hen. For each test hen, the familiar hen was placed 2 or 3 times in the left branch, and 2 or 3 times in the right branch of the Y-maze. The side on which the familiar hen was on the first testing day was balanced on group level.

The test started with an experimenter opening the guillotine door of the start box, and lasted for five minutes. The latency to visit and the total duration of the visits were scored. A test hen was considered visiting a stimulus hen when its full head and neck had entered the corresponding choosing zone. From there, the latency to visit was calculated as follows: *latency to visit the unfamiliar hen – latency to visit the familiar hen*. The data could then range from -300 to 300 (s). The visit duration was calculated as follow: *visit duration to the familiar hen / (visit duration to the familiar hen + visit duration to the unfamiliar hen) * 100*. The data could then range from 0 to 100 (%). For easy reading, instead of relative latency and visit duration proportion, these parameters will still be referred to as latency and visit duration. The individuals that did not visit any of the stimuli hens were excluded from the analyses for both the latency to visit and the visit duration in the SRT.

A longer latency to visit the familiar (unfamiliar) hen and a shorter visit duration to the unfamiliar (familiar) hen were associated with an increased interest in the unfamiliar (familiar) hen. The preference was hypothesized to depend on the hybrid, with the DW hens expected to show more interest in the unfamiliar hen compared to ISAb hens (Hewlett & Nordquist, 2019).

Chickens are known to have a strong hierarchical structure (Gottier, 1968). To avoid any bias from that perspective, the stimulus hens of each test hen were changed daily. All hens were both used as test and stimulus hens. Four hens (one per group) per day were stimulus hens first, and test hens at the end of the day. All other hens were test hens first, and then stimulus hens.

Here again, the trials were analyzed separately, as learning and habituation-sensitization effects through repetition were possible and could have influenced the results if the data were pooled (Blumstein, 2016). As for the holeboard test, light-incubated hens were expected to show better learning than dark-incubated hens (Chiandetti & Vallortigara, 2009), demonstrated by a steeper learning curve. No hybrid difference was expected in terms of learning (Dudde et al., 2018; Hewlett & Nordquist, 2019). There are no published studies on habituation-desensitization in chicken behavior, so we did not formulate any expectation in this regard.

Statistical Analyses

The statistics were performed on R Studio Desktop 2021.09.1 Build 372 (R Core Team, 2021). 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. The models were all first built with the hybrid, the incubation

treatment, their two-way interaction, and the round (when relevant) as fixed factors. When the interaction was not significant, it was removed from the models. Although the observational unit was the individual chicken, the pen was corrected for as a random effect. For the main effects, only the outcome from the final models is 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 the *p*-values are reported. An effect was considered significant if the 95% CI did not include 0 (and if $p < .05$).

The visit duration to stimuli hens in the SRT was analyzed with a t-test comparing the observed percentage to the percentage expected by chance (50%). One t-test per hybrid*incubation treatment and per trial was performed. As a result, round and interaction effects were not included in the analysis.

Table 2

Statistical Models Used To Analyze Each Parameter

Test	Parameter	Model	Distribution
	Total TH intensity	Linear Mixed-Effect Model	Gaussian
	TH intensity lateralization	Linear Mixed-Effect Model	Gaussian
	Total DCX intensity	Linear Mixed-Effect Model	Gaussian
	DCX intensity lateralization	Linear Mixed-Effect Model	Gaussian
DT	Lateralization index	Linear Mixed-Effect Model	Gaussian
HBT	Working memory	Linear Mixed-Effect Model	Gaussian
	Latency to revisit	Survival analysis	Lognormal (1,2,3,5) Exponential (4)
	Test duration	Survival analysis	Lognormal (1,4,5) Loglogistic (2,3)
SRT	Latency to visit	Linear Mixed-Effect Model	Gaussian
	Visit duration	t-test (mu = 50)	

Note. DT = detour test. HBT = holeboard test. SRT = social recognition test. Because the HBT and the SRT were performed over 5 trials, 5 analyses were conducted on each parameter. Sometimes, the data of different trials had different distributions. This is indicated with the trial numbers between brackets in the "Distribution" column. When no numbers are mentioned, all trials had the same distribution.

Results

Brain

Detailed results of this section can be found in Table S2.

Tyrosine Hydroxylase (TH)

Light-incubated chicks tended to have a more lateralized TH intensity than dark-incubated chicks in the LSt ($mean_{L:D} = -1.46$, $mean_{D} = 1.28$, $p = .09$), but that difference was not significant. There was no significant effect of the light during incubation on TH intensity lateralization in the other AOIs (p's $\geq .38$). The TH intensity lateralization was not influenced by interaction between incubation and hybrid (p's $\geq .12$), hybrid (p's $\geq .20$), sex (p's $\geq .15$), or round (p's $\geq .12$).

The total TH intensity was not influenced by the incubation treatment (p's $\geq .30$) or the interaction between incubation and hybrid (p's $\geq .26$) in any of the AOIs. DW chicks had a significantly lower TH intensity than ISAb chicks in the LSt (est = -35.45, $p = .004$, 95% CI = [-58.28; -12.66]), but not in the other AOIs (p's $\geq .13$). Male chicks had a significantly lower TH intensity than female chicks in the MSt (est = -28.30, $p = .007$, 95% CI = [-47.66; -8.57]), but not in the other AOIs (p's $\geq .15$). The total TH intensity was significantly lower in round 2 than in round 1 for all the AOIs (p's $\leq .02$).

DoubleCortin (DCX)

Dark-incubated chicks tended to have a more lateralized DCX intensity than light-incubated chicks in the LSt ($mean_{L:D}$ = -0.77, $mean_D$ = -5.27, p = .07), but that difference was not significant. Light during incubation did not influence DCX intensity lateralization in the other AOIs either (p's ≥ .30). The DCX intensity lateralization was not influenced by interaction between incubation and hybrid (p's ≥ .19), hybrid (p's ≥ .37) or sex (p's ≥ .20). Chicks from round 2 had a significantly more lateralized DCX intensity than chicks from round 1 in the MSt ($mean_1$ = -0.65, $mean_2$ = -17.98, p < .001) and the BST ($mean_1$ = 0.94, $mean_2$ = -17.52, p < .001), and also tended to, although not significantly, in the LSt ($mean_1$ = 0.19, $mean_2$ = -6.14, p = .06). There was no round effect on DCX intensity lateralization in the amygdala (p = .44).

The total DCX intensity was not influenced by incubation treatment (p's ≥ .33), interaction between incubation and hybrid (p's ≥ .51), hybrid (p's ≥ .28) or sex (p's ≥ .70). The total DCX intensity was higher in round 2 than in round 1 for all the AOIs (p's ≤ .007).

Detour Test

Light-incubated chicks were significantly more lateralized towards the left than dark-incubated chicks (est = -23.88, p = .037, 95% CI = [-46.09; -1.67]) (Figure 2). There was no significant effect of the interaction between hybrid and incubation on the lateralization index (est = -5.08, p = .83, 95% CI = [-50.40; 40.16]). There was no difference in the lateralization index of DW and ISAb chicks (est = -1.19, p = .96, 95% CI = [-45.69; 43.28]).

Holeboard Test

The detailed statistical outcomes of the holeboard test are available in Tables S3 and S4.

Working Memory

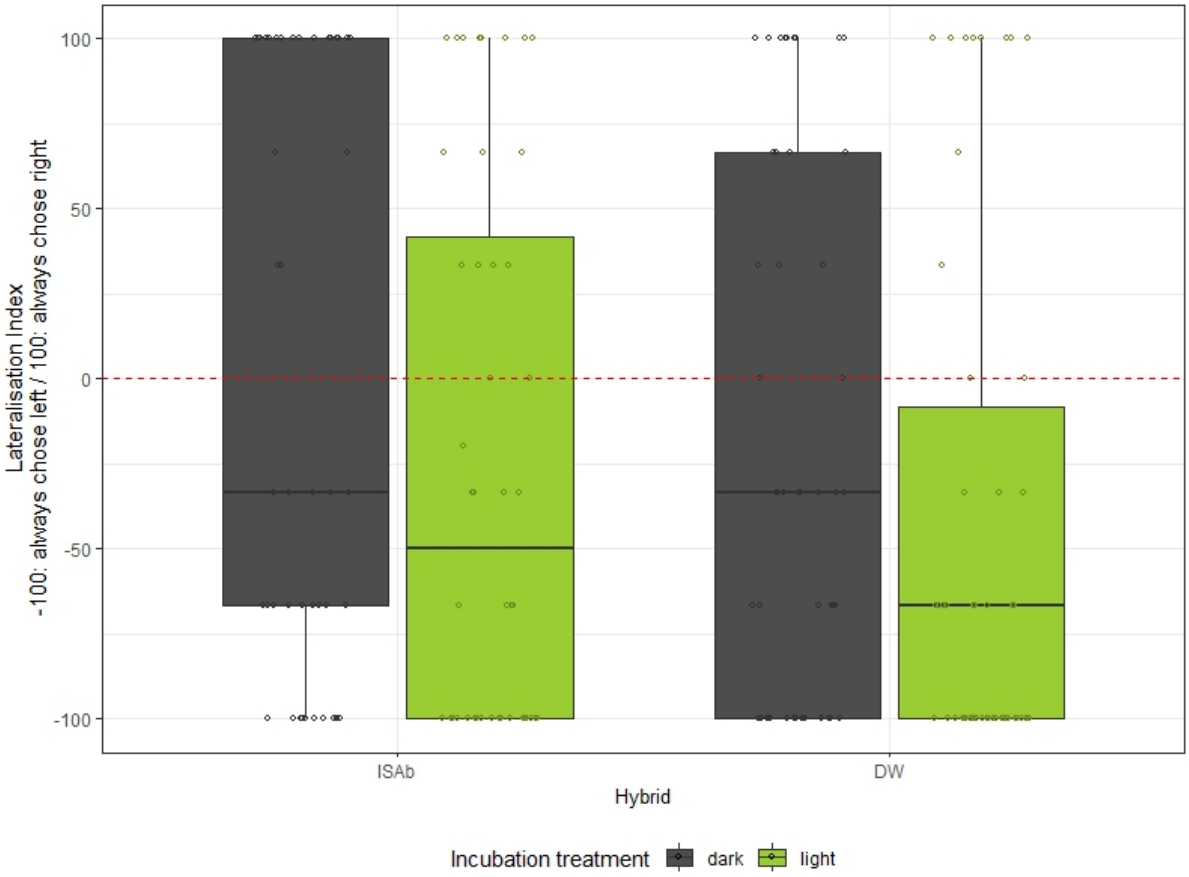
Working memory was first analyzed with the hybrid, incubation treatment and round as fixed factors, separating the data per trial (Figure 3A). There was no significant effect of incubation treatment (p's ≥ .45), nor interaction between incubation and hybrid (p's ≥ .09), nor hybrid (p's ≥ .34) on the working memory of the hens in the holeboard test. There was a significant round effect in trial 5 (est = -.37, p < .001, 95% CI = [-0.50; -0.24]), but not in the other trials (p's ≥ .12).

Working memory was then analyzed to compare the trials to each other, while still including hybrid, incubation and round as fixed factors in the model. Because the interaction was never significant in the first analysis, it was left out of this model too. Finally, because incubation treatment and hybrid had no effect on the working memory index, the analysis was performed on the entire dataset, rather than on the dataset split per treatment.

The working memory of hens in trial 1 was (respectively) .12, .14 and .15 unit lower than that of hens in trials 2, 3 and 4 (p's < .001), and .07 unit higher than in trial 5 (est = -.07, p = .05, 95% CI = [-0.14; -0.001]). The working memory of hens in trial 5 was (respectively) .19, .21 and .22 unit lower than that of hens in trials 2, 3 and 4 (p's < .001). The working memory of hens in trials 2, 3 and 4 did not differ significantly between trials (p's ≥ .47).

Figure 2

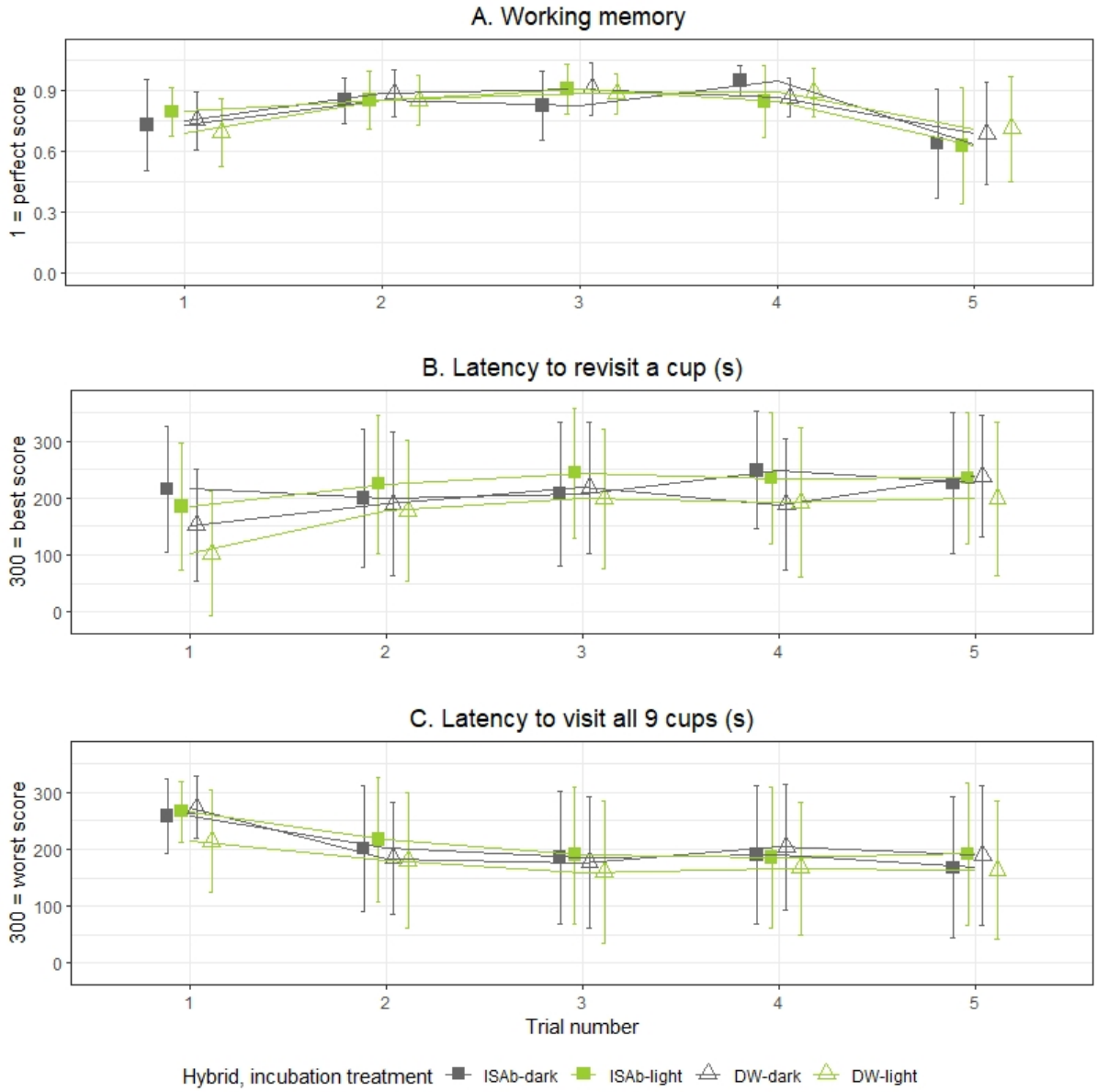
Lateralization Index During a Detour Test



Note. A lateralization index of 0 means the left and right sides were equally chosen. The closer to -100, the more often the left side was chosen. The closer to +100, the more often the right side was chosen. The distribution of individual data is shown with the dots. ISAb = ISA Brown. DW = Dekalb White. Dark = incubation in complete darkness. Light = incubation in a cycle of 12L:12D. Sample sizes: ISAb-dark = 45, ISAb-light = 48, DW-dark = 50, DW-light = 54.

Figure 3

Results from the Holeboard Test



Note. In averages ± standard deviation per trial. A: working memory score. The data of the hens that visited less than 7 cups were excluded from the analyses and the figure. Sample sizes: Trial 1: ISAb-dark = 9, ISAb-light = 9, DW-dark = 10, DW-light = 13. Trial 2: ISAb-dark = 11, ISAb-light = 10, DW-dark = 15, DW-light = 13. Trial 3: ISAb-dark = 10, ISAb-light = 10, DW-dark = 14, DW-light = 13. Trial 4: ISAb-dark = 11, ISAb-light = 10, DW-dark = 13, DW-light = 13. Trial 5: ISAb-dark = 10, ISAb-light = 10, DW-dark = 13, DW-light = 13. B: Latency to revisit a cup. C: latency to finish the test (i.e., visit all 9 cups). In B and C, if the behavior did not occur, the latency was set as 300 and included in the analyses and figures. Sample sizes: Trials 1 to 4: ISAb-dark = 19, ISAb-light = 20, DW-dark = 20, DW-light = 19. Trial 5: ISAb-dark = 18, ISAb-light = 20, DW-dark = 20, DW-light = 19.

Latency to Revisit

The latency to revisit of light-incubated hens was significantly shorter in trials 1 (est = -0.25, $p <$ .001, 95% CI = [-0.38; -0.13]) and 4 (est = -0.18, $p <$ .001, 95% CI = [-0.70; 0.34]) but longer in trial 3 (est

= 0.67, $p < .001$, 95% CI = [0.36; 0.98]) compared to dark-incubated hens (Figure 3B). They tended to have a longer latency to revisit in trial 5 too (est = 0.53, $p = .05$, 95% CI = [-0.004; 1.06]). The incubation condition did not affect the latency to revisit in trial 2 (est = 0.10, $p = .76$, 95% CI = [-0.53; 0.73]).

The interaction between hybrid and incubation treatment had a significant effect on the latency to revisit a cup in trials 1, 3 and 5 (p's $\leq .05$). More specifically, ISAb-light had a shorter latency to revisit in trial 1 (est = -0.25, $p < .001$, 95% CI = [-0.38; -0.13]), but longer (52.14s) in trials 3 and 5 (p's $\leq .03$), than ISAb-dark. DW-light hens had a significantly shorter latency to revisit in trials 1 (est = -0.70, $p < .001$, 95% CI = [-0.84; -0.56]) and 3 (est = -0.60, $p = .005$, 95% CI = [-1.03; -0.18]) than DW-dark (respectively 0.70 and 56.48s).

DW hens had significantly shorter latencies to revisit than ISAb hens in trials 1 (est = -0.63, $p < .001$, 95% CI = [-0.82; -0.43]) and 4 (est = -1.01, $p < .001$, 95% CI = [-1.57; -0.45]), but not in the other trials (p's ≥ 0.15).

The hens from round 1 had a significantly longer latency to revisit than the hens from round 2 in every trial (p's $< .001$).

Latency to Finish the Test

Light-incubated hens finished the holeboard test significantly later than dark-incubated hens in trial 2 (est 0.49, 95% CI = [0.17; 0.82], $p = .003$), but not in the other trials (p's $\geq .53$) (Figure 3C).
The latency to finish the holeboard test was significantly influenced by the interaction between hybrid and incubation in trials 1 and 2 (p's $\leq .049$). More specifically, DW-light hens were faster to finish the test in trial 1 compared to DW-dark (est = -0.60, $p < .001$, 95 CI % = [-0.85; -0.35]). ISAb-light took longer to finish the test in trial 2 (est = 0.49, $p = .003$, 95% CI = [0.17; 0.82]) compared to ISAb-dark. The interaction between hybrid and incubation did not influence the other trials (p's $\geq .10$).

DW hens finished the test significantly earlier than ISAb hens in trial 1 (est = 0.25, $p < .001$, 95% CI = [0.12; 0.39]), but not in the other trials (p's $\geq .19$).

Hens from round 1 took significantly longer to finish the test in all trials compared to round 2 (p's $< .001$).

Social Recognition Test

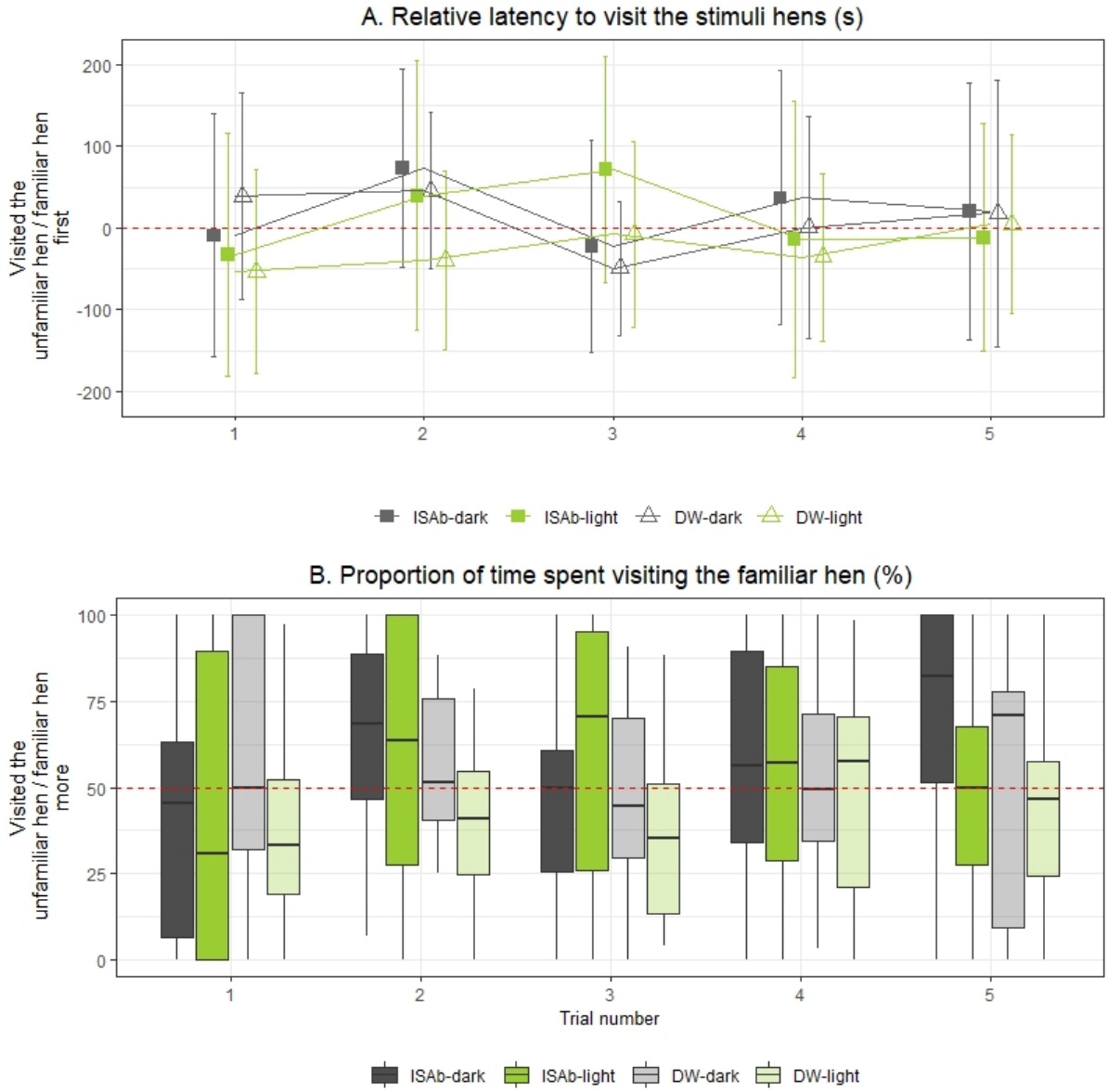
The detailed statistical outcomes of the social recognition test are available in Tables S5 and S6.

Latency to Visit the Stimuli Hens

The light-incubated hens were significantly faster than dark-incubated hens to visit the familiar hen in trial 3 (est = 66.24, $p = .02$, 95% CI = [11.21; 121.26]) (Figure 4A). The latency to visit the stimuli hens was not influenced by the incubation in the other trials (p's $\geq .20$). DW hens tended to visit the unfamiliar hens earlier than ISAb hens in trial 3 (est = -51.09, $p = .08$, 95% CI = [-106.21; 4.01]), although that was not significant. Hybrid did not influence the latency to visit the stimuli hens in the other trials (p's $\geq .36$). The latency to visit the stimuli hens was not influenced by the interaction between incubation and hybrid (p's $\geq .34$) or round (p's $\geq .33$) in any of the trials.

Figure 4

Results from the Social Recognition Test



Note. The data of the hens that visited none of the stimuli hens were excluded from the analyses and figures. Sample sizes: Trial 1: ISAb-dark = 17, ISAb-light = 14, DW-dark = 13, DW-light = 16. Trial 2: ISAb-dark = 18, ISAb-light = 18, DW-dark = 16, DW-light = 18. Trial 3: ISAb-dark = 17, ISAb-light = 16, DW-dark = 17, DW-light = 18. Trial 4: ISAb-dark = 17, ISAb-light = 18, DW-dark = 18, DW-light = 18. Trial 5: ISAb-dark = 16, ISAb-light = 16, DW-dark = 14, DW-light = 15. A: latency to visit the stimuli hens relative to each other, in averages ± standard deviation per trial. < 0: the hens visited the unfamiliar hen first. > 0: the hens visited the familiar hen first. B: Proportion of time spent visiting the familiar hen related to the total visit duration, per trial. < 50: the hens visited the unfamiliar hen more. > 50: the hens visited the familiar hen more.

Duration of Visit to the Stimuli Hens

ISAb-light and DW-dark spent around 50% of their visit time with each of the stimuli hens in all trials (p's ≥ .22). ISAb-dark hens spent significantly more time with the familiar hen than expected by chance in trials 2 (t_{17} = 2.29, p = .04, 95% CI = [51.22; 80.21]) and 5 (t_{15} = 2.19, p = .045, 95% CI = [50.48;

87.75]), but spent around 50% of their visit time with each of the stimuli hens in the other trials (p's ≥ .48) (Figure 4B). DW-light hens spent significantly more time with the unfamiliar hen than expected by chance in trials 1 (t_{15} = -2.37, p = .03, 95% CI = [20.41; 48.41]) and 3 (t_{17} = -2.31, p = .03, 95% CI = [23.85; 48.79]), but spent around 50% of their visit time with each of the stimuli hens in the last two trials (p's ≥ .09).

Discussion

The effects of green light during incubation on brain and cognition were investigated in Dekalb White (DW) and ISA Brown (ISAb) laying hens. More specifically, tyrosine hydroxylase (TH) and doublecortin (DCX) intensity and lateralization in the brain were investigated as proxies for dopamine production and brain plasticity, respectively. To measure cognition, detour, holeboard, and social recognition tests were performed to study behavior lateralization, working memory and social recognition, respectively. Green light during incubation had inconsistent effects on the laying hens.

Incubation Effects

There was no significant effect of green light during incubation on TH or DCX intensity lateralization. A more lateralized intensity means that one hemisphere specialized in the production of TH or DCX (Rogers, 1982). Specialization of brain regions into certain functions allows for better performance in these functions (Magat & Brown, 2009). It is interesting to note that the left hemisphere is associated with positive cognitive bias, whereas the right hemisphere, with negative cognitive bias (Rogers, 2010). Several studies linked positive cognitive bias to positive welfare (reviewed for example in Košťál et al., 2020; Mendl et al., 2009). Therefore, it would be interesting for future research to investigate TH and DCX lateralization in other areas of interest (AOIs), and whether a higher ability to produce dopamine or a higher brain plasticity in the left hemisphere could increase positive welfare further, compared to the right hemisphere.

Similarly, incubation condition did not influence total TH and DCX intensities. In contrast to our expectations, green light-incubated chicks were not prone to higher dopamine production, nor to higher brain plasticity, than dark-incubated chicks. Further research in other AOIs would be interesting, especially in AOIs involved in stress regulation or social behavior.

In the detour test, the behavior of the green light-incubated chicks was significantly more lateralized than the dark-incubated chicks, which was expected. The chicks preferred to pass the barrier *via* the left, which means they were looking at their reflection in the mirror with their right eye (controlled by the left hemisphere). The right hemisphere is specialized in recognizing familiar conspecifics, whereas the left hemisphere is specialized in distinguishing chickens from other species (Rosa-Salva et al., 2012). As a consequence, chicks preferably use their left eye (right hemisphere) to categorize stimuli, and their right eye (left hemisphere) to discriminate between familiar and unfamiliar conspecific (Rogers, 1996). The chicks in this experiment were never exposed to any mirrors before the test, meaning they may have experienced their own reflection as an unfamiliar conspecific. Approaching it with their right eye (left hemisphere) therefore seems logical. In addition, the left hemisphere is involved in approach behavior (Rogers, 2010). This confirms the validity of our results and the importance of green light during incubation for brain lateralization and specialization.

In the holeboard and social recognition tests, several significant differences were found, but were inconsistent. In the holeboard test, green light-incubated hens revisited cups sometimes earlier, sometimes later than dark-incubated hens. In the social recognition test, green light-incubated hens approached the familiar hen faster than dark-incubated hens in one of the trials, whereas the other trials showed no significant difference between the incubation treatments. These results are surprising. Indeed, green light-incubated hens were expected to have a better working memory than dark-incubated hens. However, an explanation may be sought in the testing procedures, as the working memory indexes were overall high (good), which may indicate that the test was too easy to uncover differences between incubation treatments

(see "limitations" below). In addition, green light-incubated hens were expected to have enhanced preferences in the social recognition, and ISAb hens showed the opposite. It is possible that environmental factors, such as social agitation around the test (see "limitations" below), altered the incubation effects. Another possibility is that green light during incubation, contrary to our expectations, increased the interest for the unfamiliar hen in both the hybrids, relatively speaking. Indeed, to our knowledge, previous studies investigating sociality used only dark-incubated chickens, and the effect of green light during incubation on social preferences is unknown.

There are also inconsistent results in terms of significant interaction effects in the holeboard test. ISAb-light revisited cups sometimes earlier, sometimes later than ISAb-dark. However, DW-light hens revisited cups earlier, and not later, than DW-dark. It should however be mentioned that the interaction was not significant in all the trials. The somewhat consistent results within DW hens may be due to the difference in light exposure during incubation. Indeed, light transmission is five times higher through DW eggshells than through ISAb eggshells (Manet, Kliphuis, Van Den Brand, et al., 2023). As a result, it is possible that the effect of green light during incubation was stronger in DW than in ISAb hens.

Overall, the effects of green light during incubation seem to have been stronger on the cognitive measurements than on the brain measurements. That is especially interesting considering the brains analyzed were those of one-day-old chicks, whereas the cognition tests were performed at 3-4, 21 and 24 weeks of age. Indeed, one could expect the incubation effects to fade away as the chicks grow, as with fear of humans for the same chicks (Manet et al., 2023): green light during incubation influenced the chicks' behavior significantly only at 6 weeks of age, not at 10 weeks of age or later. Based on that, stronger effects would have been expected around hatching than at adulthood. Instead, our results, combined with the results of fear of humans, suggest that green light during incubation affects specific traits, rather than a specific time period: some significant effects were found at adulthood. This is supported by several studies (e.g., Coulon et al., 2011; Jarvis et al., 2006; Nordquist et al., 2013) that showed that prenatal experiences can have long-term influence on individuals.

Other Main Effects

Significant differences in total TH intensity were found in the MSt in relation to sex, and the LSt in relation to hybrid. These brain areas are involved in learning, memory and behavioral flexibility. There is a strong link between the dopaminergic system, including TH, and cognitive functions (Tahamtani et al., 2015). Although it is not possible to directly correlate TH levels to cognitive abilities, the observed differences may indicate sex or genetic differences in cognition. The total DCX intensity was not influenced by any of the treatments, meaning all chicks had similar brain plasticity and possibly neurogenesis, based on this indicator (Capes-Davis et al., 2005).

In the holeboard test, DW revisited the cups significantly faster than ISAb in two trials, thus performing worse. ISAb hens typically eat more than DW (Hendrix-Genetics, 2020a, 2020b), which could explain their high motivation in this food-rewarded test. However, the ISAb used in this test ate less than the DW (personal observation), which does not support this explanation. It is important to note that these significant differences were smaller than 2 seconds, which does not seem biologically relevant, and will therefore not be discussed further. In the social recognition test, ISAb-dark hens spent significantly more time with the familiar hens than expected by chance on two trials, whereas DW-light spent significantly more time with the unfamiliar hens than expected by chance in two trials. ISAb were expected to show more interest in the familiar hen, and DW in the unfamiliar hen (Hewlett & Nordquist, 2019), which is in line with our results. However, we were expecting similar results for ISAb-light and DW-dark as well, which did not occur. We used adult hens, whereas Hewlett and Nordquist (2019) used chicks, which may be an explanation for our differing results. In addition, we used different hybrids. Although there are differences between white and brown hybrids (e.g., Manet et al., 2023; Uitdehaag et al., 2011), we cannot exclude the possibility that our white (brown) hybrid differed from that of the other study.

Limitations

It is worth mentioning that the dark-incubated chicks were exposed to light during the hatching checks. As mentioned in the methods, this exposure was minimal: there were 9 hatching checks, of which the first two took less than 10 seconds because no chicks had hatched yet. The remaining 7 checks took a few seconds to two minutes, meaning that the combined exposure took less than 14 minutes. That duration was not enough to affect lateralization (Rogers, 1990; Zappia & Rogers, 1983). It could be argued that the health quality checks, performed with the light turned on, could affect lateralization. However, the scope of this research was the effects of light during incubation on laying hen brain and cognition. Because the health quality checks were performed after hatching, this exposure was not considered as light exposure during incubation.

The holeboard test showed a statistically increased performance from trial 2 onwards, exhibiting a learning curve. Performance decreased in the last trial, which probably means that the hens lost interest in the food reward, rather than became worse at the task (Blumstein, 2016). Therefore, the number of trials seems to have fit this experimental set-up.

The working memory in the hens was overall good, which may indicate that the test was too easy to highlight potential differences between incubation treatments and/or hybrids, in other words, a "ceiling effect. Indeed, the bottom of the cups were visible for hens without them having to peck, as they were able to see whether they already had eaten the food rewards. Future research should focus on improving the methodology of the holeboard test for chickens by making access to the food reward more difficult, for example with lids on the cups. This would require training the animals beforehand to access the food, which would be more time-consuming. Another option would be to use a larger test arena, and count the number of times the hens approached the cup close enough to see the bottom of the cup, rather than the number of times they pecked at it. Here, the hens would need to be explorative enough to walk through the entire arena.

The results of the social recognition test are difficult to interpret for several reasons. Firstly, it is difficult to know how the hens perceived the test arena. They could approach the stimuli hens, but only to a certain extent: a wire mesh separated them from the rest of the arena. Although some physical contact was possible (e.g., pecking), it could only happen if the test hen approached; stimuli hens did not have control on this respect. This offers a certain security to visit the stimuli hens, even if it is an unfamiliar or dominant hen. Hence, the motivation cannot be pinpointed with certainty: after experiencing the safety of the situation, hens could be motivated to visit a more intimidating hen than they would have in a less safe situation. Secondly, there was a lot of social agitation in home pens during the time period of this test. Fights in the ISAb-light group resulted in comb damage and head wounds, which led to some birds being socially isolated to recover. Because this occurred only in this group, it is difficult to determine if group differences might also be due to the fighting rather than the treatment itself. These birds were excluded from the test, but the included animals were likely also impacted by the stress of the fights, and their social interactions may have been impacted. It is possible that these negative social interactions were due to the age of the hens: 24 weeks old is at the beginning of sexual maturation, and hens' aggressivity usually increases around that age (Craig et al., 1975).

Finally, significant round effects were observed in several of the parameters described in the present paper, although no clear pattern emerged from it. This highlights the difficulty of reproducibility in animal science, and the need to design experiments carefully, and repeat experiments as much as possible to obtain results that can reliably be translated to practice.

Conclusion

To conclude, green light during incubation had no significant effects on tyrosine hydroxylase nor DoubleCortin in chick MSt, LSt, BST nor amygdala and significant effects on chick lateralization and adult cognitive capacities, in ISA Brown and Dekalb White laying hen hybrids. Further brain analyses should be performed in other areas of interests related to stress and social behavior to estimate whether green light

during incubation could help laying hens to adapt better to their environment, for example when changing facility from the rearing phase to the laying phase. Similarly, more cognition tests should be performed at different stages of the laying hens' lives to give a larger overview of the effects of green light during incubation on laying adaptive and cognitive capacities.

Acknowledgements

The authors thank Elly Zeinstra, Judith Hendriks and Dagmar Jongenelen for performing the lab work on the brains, and Dr Richard Wubbolts for providing an adapted script to measure TH and DCX intensity in ImageJ/FIJI.

The authors thank Dagmar Jongenelen for drafting the supplementary protocol.

The authors are grateful to the colleagues and students who helped collecting the brains and/or cognition data (in alphabetical order): Serge Alindekon, Dewi Bouman, Inge van der Burg, Viviane Carvalho, Casper van Eekelen, Dylan Geerman, Klara Grethen, Judith Hendriks, Kirsten Hoppener, Marjolein Jongerius, Elsemieke van der Laan, Zinzhi van Leeuwen, Lisette Martens, Britta Mescher, Arjen van Putten, Dronika Soedhoe, Isabelle Spierings, Lisa Veldkamp, Marenne Vis, Jary Weerheijm, Vivian Witjes and Claudia van der Zijden.

Author Contributions: 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 the article critically for important intellectual content. **TB Rodenburg:** proposal writing and funding acquisition, conception and design of the study, analysis and interpretation of data, revising the article critically for important intellectual content. **VC Goerlich:** conception and design of the study, analysis and interpretation of data, revising the article critically for important intellectual content. **FAM Tuyttens:** proposal writing and funding acquisition, analysis and interpretation of data, revising the article critically for important intellectual content. **RE Nordquist:** conception and design of the study, analysis and interpretation of data, revising the article critically for important intellectual content.

Funding: This project received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 812777. This document reflects only the authors' view and the European Union's Horizon 2020 research and innovation program is not responsible for any use that may be made of the information it contains.

Conflict of Interest: The authors declare no conflict of interest.

Data Availability: The raw data is available here: doi.org/10.5281/zenodo.14920238

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Supplementary materials: available here: https://zenodo.org/records/14920251