Poultry Science

The impact of early-life conditions on visual discrimination abilities in free-ranging laying hens

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ABSTRACT Conditions during incubation and rearing can greatly affect the developmental trajectory of chickens, in a positive and negative way. In this study, the effect of early-life conditions on the visual discrimination abilities of adult, free-ranging laying hens was examined. These early-life treatments entailed incubation in a 12/12h green light/dark cycle and rearing with Black soldier fly larvae (**BSFL**) as foraging enrichment. Through a modified pebble-floor test, 171 hens of 41 to 42 wk old, housed in mobile stables with outdoor access, were tested for their ability to discriminate between food and nonfood items (mealworms and decoy mealworms). Each hen was allowed 60 pecks during the trial, from which the overall success rate, as well as within-trial learning was investigated. The latter was accomplished by dividing the 60 pecks into 3 blocks of 20 pecks and comparing the success rate between these blocks. Due to another ongoing experiment on range use, roughly half the hens received range enrichment (mealworms) at the time of testing, so this was included as a covariate in the analysis. Incubation with green light did not have an effect on the visual discrimination abilities of adult laying hens. Rearing with BSFL did have a limited beneficial effect on the visual discrimination abilities, as evidenced by a higher success rate during the first block of the visual discrimination trial. These enhanced visual discrimination abilities might be useful in a more complex free-range setting, where the animals have more foraging opportunities. Hens that received range enrichment at the time of testing, also had a higher success rate during the visual discrimination test, though they had a lower degree of test completion, likely due to habituation to the mealworms as an enrichment. The positive effects of BSFL during rearing and mealworms during the laying period stress the importance of enrichment throughout the life of the hens.

Key words: laying hen, early-life, enrichment, black soldier fly, visual discrimination

2024 Poultry Science 103:104236
https://doi.org/10.1016/j.psj.2024.104236

INTRODUCTION

It has been established that early-life conditions − both during incubation and rearing − are a potent factor in the later development of chickens (*Gallus gallus domesticus*) (Bateson, 1979; Janczak and Riber, 2015). Positive or negative experiences can have long-term effects, that often persist into adulthood (De Haas et al., 2021; Riber et al., 2007; Rodenburg et al., 2008). In the case of laying hens, negative early-life experiences (including lack of stimulation during rearing) may result in the display of undesirable behaviors during the laying phase, such as feather pecking (Blokhuis and Van der Haar, 1992; Huber-Eicher and Wechsler, 1997; Johnsen et al., 1998). On the other hand, positive early-life experiences could promote favorable behaviors, such as foraging, potentially improving overall welfare (Blokhuis and Van der Haar, 1992; Janczak and Riber, 2015). As feather pecking is considered a maladaptive behavior stemming from a lack of foraging opportunities (e.g. feed and ground pecking), the incidence of feather pecking behavior and foraging behavior are often inversely related on a group level (Blokhuis, 1989; Huber-Eicher and Wechsler, 1997; Rodenburg and Koene, 2003; Rodenburg et al., 2008; van Hierden et al., 2002).

Considering the role of the visual system in guiding chicken behavior, well-developed visual skills are likely

Received June 18, 2024.
Accepted August 14, 2024.

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important for efficient foraging behavior (Freire, 2020; Rogers and Kaplan, 2019). This is especially true for chickens in free-range systems, where the foraging opportunities and the general—outdoor—environment are more complex compared to (conventional) indoor systems. Indeed, visual skills are of great importance in order to find food and to distinguish between food and nonfood items, but are also key for e.g. predator avoidance or social interaction.

Studies have shown that light exposure during incubation can affect behavior in later life, likely through an increased lateralization of the brain (e.g. Archer and Mench, 2017; Rogers et al., 2007). To understand the effect of light during incubation, one needs to go back to the wild ancestors of domestic chickens, Red Junglefowl (*Gallus gallus*), where the mother hen would occasionally leave the nest in search of food during the final days of incubation (though only for about 20 min per day), and during these short periods would expose her eggs to daylight (Mrosovsky and Sherry, 1980). Due to the embryo's position in the egg at the end of incubation, only the right eye is exposed to light from outside the eggshell. This asymmetrical light exposure affects the lateralization of the avian brain and stimulates the right-eye system (i.e. the right eye and left-brain hemisphere), resulting in a more dominant left hemisphere (Rogers and Sink, 1988; Rogers, 1982). Hence, the period during incubation from d 18 on is referred to as the sensitive period for the development of the visual pathways (Rogers, 1996). In addition, the asymmetrical light exposure affects the functioning of both hemispheres as it alters the balance of control between the hemispheres by modulating interhemispheric interactions (Letzner et al., 2014; Letzner et al., 2017; Manns and Römling, 2012). Increased brain lateralization is associated with enhanced task performance, such as distinguishing food from 'distracting' nonfood items (traditionally often pebbles) (Güntürkün et al., 2000; Rogers and Kaplan, 2019). Furthermore, several studies (mainly on broiler chicks) have shown that chickens with a stronger lateralized brain are less sensitive to environmental stressors, as they are better able to control fear responses initiated by the right hemisphere (Rogers, 2010; Rogers and Kaplan, 2019). However, in that regard there are inconsistencies regarding the effect of light during incubation, where both an increase (Dimond, 1968) and a decrease (Archer, 2017; Archer and Mench, 2014a) in fearfulness have been reported. In this context, the color of the light emerges as a potentially crucial factor. Notably, green light exhibits promise to enhance welfare in laying hens, as evidenced by the study conducted by Özkan et al. (2022), showcasing its efficacy in reducing feather pecking.

After hatching, rearing the chicks in a complex environment increases the likelihood that they will develop the necessary skills to navigate in a complex environment later in life (Blokhuis and Van der Haar, 1992; Gunnarsson et al., 2000). For example, early exposure to varied stimuli or enrichment has been shown to stimulate brain development, as well as reduce fearfulness (Brantsæter et al., 2016; Campbell et al., 2019; de Haas et al., 2014; Jones, 1982; Reed et al., 1993). A study by Blokhuis and Van der Haar (1992) even found that providing grain as a pecking incentive during rearing had effects that lasted until the laying phase, with more efficient food pecking and less feather pecking. Supplying live black soldier fly larvae (**BSFL**, *Hermetia illucens*) is particularly appealing, also from an economic perspective. They possess the ability to consume organic side streams, have an interesting nutritional profile (Ewald et al., 2020; Giannetto et al., 2020; Schmitt et al., 2019) and their supplementation into the diet of the chicken can result in reduced feed costs without adversely affecting performance or egg quality (Lokaewmanee et al., 2023). Furthermore, in their natural environment, chickens dedicate a significant amount of time to searching for and consuming live insects. As such, access to insects might positively contribute to chicken welfare. Indeed, potential welfare benefits − such as an increase or change in activity/foraging behavior, and better feather quality − have been demonstrated in broiler chickens and/or laying hens (Ipema et al., 2020; Kliphuis et al., 2023; Star et al., 2020; Tahamtani et al., 2021). So far, it is less clear whether supplying live BSFL during rearing has a lasting effect during the laying phase. It seems possible that the provision of live insects during the rearing phase helps with the development of their visual discrimination abilities, thus in later life contributing to a more efficient foraging behavior, especially in a free-range environment.

This study is part of a larger study looking into the effect of early-life conditions (incubation with green light and rearing with BSFL) on the different life stages of laying hens. Though green light does not seem to affect hatching characteristics (Manet et al., 2023b), it does seem to slightly reduce fearfulness (though only towards humans) during the rearing stage (Kliphuis et al., 2024; Manet et al., 2023a). Interestingly, enrichment with BSFL seems to result in more frequent, but shorter foraging bouts by the pullets (Kliphuis et al., 2023).

Given the influence of early-life conditions on the developmental trajectory of hens and its potential effects during the laying phase, this study looks into the longer-term impact of light during incubation and the provision of BSFL during rearing. This study aims to shed light on how these treatments may affect visual discrimination abilities of hens during the laying phase, as these are crucial for efficient foraging behavior, which is particularly useful in a more challenging free-range environment. We also evaluate the effect of enrichment with larvae at the time of the discrimination task, as roughly half of the hens received larvae enrichment at the time of testing as part of an ongoing range use study. More specifically, we hypothesize that: (1) hens that were incubated with green light possess better visual discrimination abilities during the laying phase, due to a more lateralized brain; (2) hens that received BSFL during the rearing phase (and were hence reared in a more complex environment), possess better visual discrimination abilities during the laying phase; (3) the combined effect of these 2 treatments is larger than effect of the individual treatments; (4) hens that received mealworms during the laying phase would perform better in a discrimination task, but may also loose interest in the larvae faster.

MATERIALS AND METHODS

Ethical Statement

Incubation and rearing. The study received approval from the Dutch Central Authority for Scientific Procedures on Animals (CCD) under license number AVD1080020198685 and from the Animal Welfare Body Utrecht under work protocol numbers 8685-1-01 and 8685-1-03. The research adheres to Dutch legislation and complies with the EU directive on animal experimentation.

Laying. Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Ethics Committee of Animal Experimentation, ILVO, Belgium (approval number 2020/368).

Incubation Conditions

A total of 600 eggs from the layer hybrid ISA Brown (Hendrix Genetics, sourced from the 'Het Anker' hatchery in Ochten, the Netherlands) were incubated at the CARUS facility of Wageningen University & Research (Wageningen, the Netherlands) in April 2021. The eggs were randomly assigned and subjected to 2 incubation conditions: light (**Inc+**) or dark (**Inc-**) incubation. Inc- eggs were not exposed to any light during incubation, whereas Inc+ eggs were exposed to 12 h of green light and 12 h of darkness daily from d 0 to d 21 of incubation. Light was provided by green LED strips (520 nm), emitting 400 lux at egg level. After hatching, female chicks underwent a quality and health check using the protocol described in Heijmans et al. (2022) and received neck labels for individual identification, while surplus male and female chicks were euthanized using cervical dislocation. Beak trimming was not performed. For more details on the incubation process, we refer to Kliphuis et al. (2023).

Rearing Conditions

At the age of 1 d, a total of 200 female chicks were transported to Utrecht, where they were housed at the Farm Animal research facility of the Faculty of Veterinary Medicine at Utrecht University (Utrecht, The Netherlands) throughout the rearing phase, up to 19 wk of age.

Chicks were housed in 24 rearing pens, with dimensions 246×88×241 cm (length x width x height). Pens were separated by wire mesh and a 60 cm high wooden barrier to prevent visual contact between adjacent pens. The pen floors were covered with wood shavings. The pens were equipped with a heat lamp, perches, a water bucket featuring drinking nipples, and a round feeder. Heat lamps were employed to maintain a temperature of 35°C on d 1, with height adjustments made over the following days to lower the temperature in the pens. Room temperature was gradually decreased from 25°C on d 1 to 18°C by the fifth wk of age. Lighting was facilitated by vertical high-frequency dimmable bird lights (GlassLux Standard 1 × 36W Philips), and natural daylight entered the stable through skylights with automated hatches (Boon Agrosystems), enabling control over daylight hours. The number of light hours was gradually reduced from 23 h on d 1 to 12 h by the fifth wk of age (standardization of the light-dark cycle). To minimize strong responses to environmental noise and human presence, a radio played classical music in the stable 24/7 throughout the entire rearing period.

The birds underwent vaccinations following a standard Dutch/Belgian vaccination regimen. In the initial week, the chicks were provided with standard rearing feed (mixed grain, Starter 1, De Heus, Ede, The Netherlands, see also Supplementary file S1). From d 7 onward, tailor-made feed (meal) from Research Diet Services gradually replaced the standard feed. Also starting from d 7, half of the Inc+ and half of the Inc- pens had chicks that were given live BSFL (Bestico, Berkel and Rodenrijs, The Netherlands), while the remaining chicks received no larvae (**Lv+** and **Lv-**, respectively). The quantity of larvae provided corresponded to 10% of the daily nutritional requirement, as recommended by the ISA Brown product guide. Chicks in pens without larvae received feed with a 10% BSFL replacer to eliminate any nutritional effects attributed to larvae consumption. Details on the composition and nutritional value of the diets can be found in Supplementary files S2 and S3. The BSFL were presented in transparent cylinders (15 × 4 cm) with three 9 mm holes each. This design was adapted from a previous study conducted with broilers (Ipema et al., 2020), and a pilot study confirmed the design was also suitable for layer chicks (Kliphuis et al., 2023). Two dispensers per pen were supplied 6 d per week, spanning from 1 to 19 wk of age.

Laying Conditions

At 17 wk of age, 5 hens per treatment were sacrificed for brain collection for a related project (ChickenStress, analysis in progress). In addition, 1 pullet was euthanized at 4 wk of age because of severe growth retardation. At 19 wk of age, the remaining 179 hens were transported to the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO, Merelbeke, Belgium), where they stayed between September 2021 and August 2022. They were housed in 2 purpose-built mobile housing units, each divided into 2 compartments by a central wall, resulting in a total of 4 stable compartments with a floor area of 9.6 m^2 each. The floors of the stable compartments were covered with wood shavings. In each of these 4 compartments, a maximum of 50 hens could be housed (yielding an indoor space allowance of 0.19 m^2 per hen). The hens were housed in 4

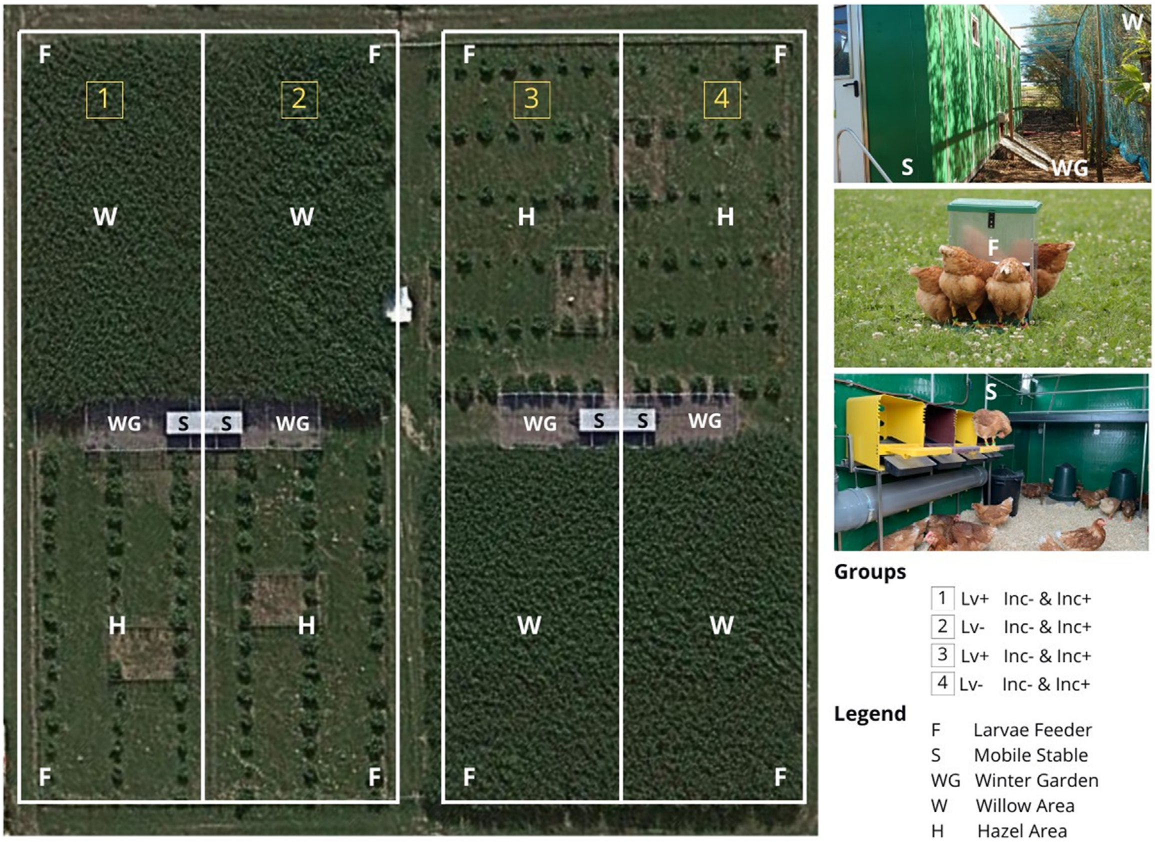

Figure 1. Laying conditions. Left panel – Overview field. Right panel top to bottom – Mobile stable in winter garden; chickens using larvae feeder; inside of mobile stables. Lv+: rearing with BSFL; Lv-: rearing without BSFL; Inc+: incubation with green light; Inc-: dark incubation.

compartments (laying groups), 2 for Lv+ hens and 2 for Lv- hens. To maintain statistical power with only 4 laying groups, each group comprised a mix of Inc+ and Inc- hens (Figure 1). Due to an error in randomizing the treatments in the rearing stage (for details, see Kliphuis et al., 2023), the number of Inc+ and Inc- hens per laying group were unequal. In addition, during intake, 4 hens were placed into an incorrect laying group, and as this was discovered a few weeks into the round after the acclimatization period, it was decided to leave the hens in their existing groups rather than disturb their social environment, leading to an imbalance in the laying groups. Specifically, 3 hens from laying group 1 were placed in group 2 and 1 hen from laying group 3 was placed in group 4. All hens could be individually recognized (including their incubation treatment) by a combination of color-coded leg bands.

Each compartment contained 10 nipple drinkers (5 on each side; 1 nipple per 5 hens) and 2 circular tower feeders, each with a circumference of 150.8 cm (6 cm per hen). Each stable also contained 8 nest boxes (4 per side; 1 per 6.25 hens), and 4 plastic perches, each being 2.4 m long (19.2 cm per hen) and mounted above a removable manure tray. The hens received organic feed from Bio'Or (Poperinge, Belgium), the detailed composition of the feed can be found in Supplementary files S4 and S5.

Each group had access to a 22×88 m (1936 m^2) outdoor run, with half of it being relatively open terrain featuring young, sparse hazel trees (6-meter-spaced rows every 3 meters, planted April 2017) with grass underneath, and the other half densely covered by short-rotation coppice of willow (2-meter-spaced double-rows of willows planted approximately every meter, planted April 2013). The range was accessible through 2 popholes of 40×50 cm (W x H), one on each side of the compartment. The popholes opened and closed automatically according to a programmed schedule (opening at 8 a.m., closing 45 min after sunset). During the period of mandatory confinement due to avian influenza (15th November, 2021–14th May, 2022), the hens did not have access to the entire outdoor run, only to a smaller winter garden. This winter garden was covered with nets and had an approximate size of 72 m^2 per group.

To test the effect of range enrichment during the laying period on ranging behavior (to be published elsewhere) 2 12 kg-capacity operant feeders filled with live mealworms were placed outdoors, in a crossover-crossback design. One feeder was positioned at each end of the run in the different vegetation types. Mealworms were provided on the outdoor range of 2 of the 4 flocks daily for a period of 12 wk (4 kg per feeder, twice per week), after which the treatment crossed over to the other 2 groups. This resulted in—at any given time—2 groups (1 Lv+ and 1 Lv-) receiving mealworms (**EnR+**) and the other 2 groups not receiving mealworms (**EnR-**). At the time of the visual discrimination trial, laying groups 1 and 2 received mealworms, while groups 3 and 4 did not. The EnR+ groups in our study were in

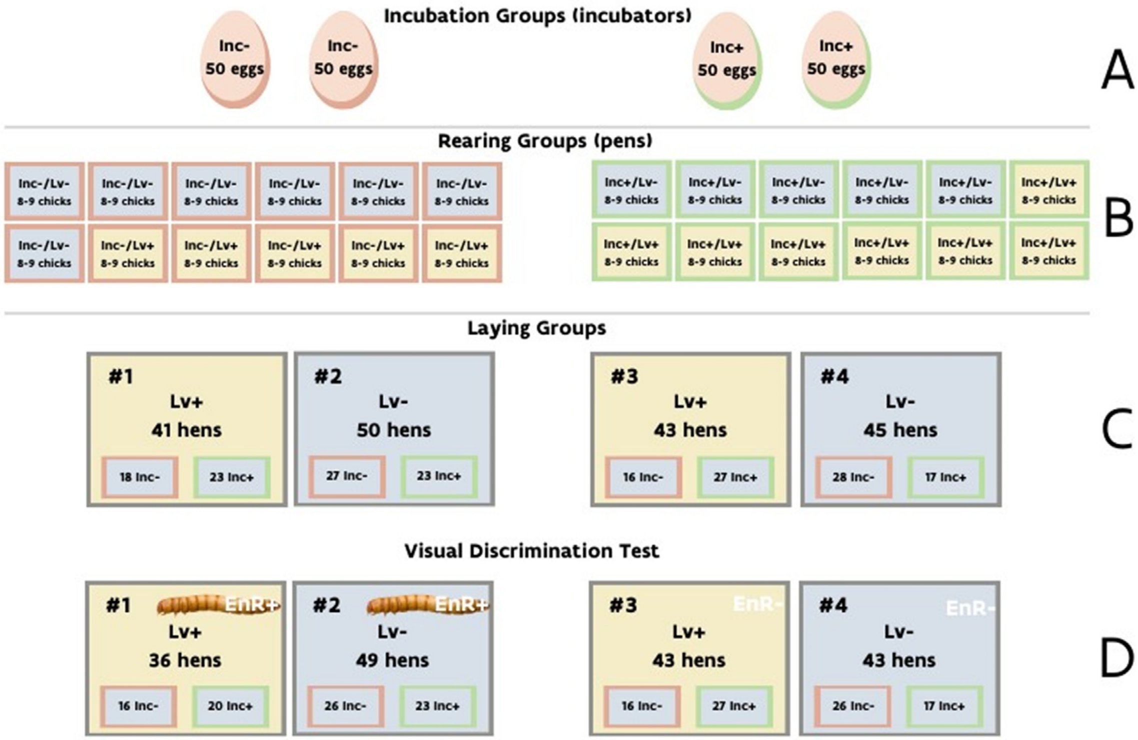

Figure 2. Layout of the experimental groups. (A) Incubation groups (200 selected eggs), (B) rearing groups, (C) laying groups, (D) visual discrimination test. Inc+: incubation with green light; Inc-: dark incubation; Lv+: rearing with BSFL; Lv-: rearing without BSFL; EnR+: range enrichment at the time of testing; EnR-: no range enrichment at the time of testing. Due to a mistake during the intake process, 3 hens from group 2 and 1 hen from group 4 were Lv+.

wk 10-11 (out of 12) of their range enrichment period. Though not a research question in the present study, whether a chicken received range enrichment or not (EnR+ or EnR-) at the time of the visual discrimination test could have affected their performance in trial. For a schematic overview of number of individuals in each incubation, rearing and laying group, as well as the individuals present and tested for the visual discrimination test, see Figure 2.

Visual Discrimination Test

The testing took place in February 2022 (when the hens were 41-42 wk old) at the mobile stables at ILVO. At that time, 171 hens were still present, see Figure 2. All these individuals were tested during the visual discrimination test (ATOL 0000924). The number of hens were more or less equally divided among early-life treatments and range enrichment: Inc- and Inc+: 84/ 87, Lv- and Lv+: 92/79, EnR- and EnR+ 86/85. As mentioned in the previous section, laying groups were already unequal at the start of the laying phase, though this inequality was exacerbated by a fox attack in laying group 1 (leading to an even smaller number of hens in that group). Consequently, stocking density differed somewhat among laying groups. However, it is unlikely that this rather small difference in stocking density would affect the hens' visual discrimination abilities.

The protocol was based on the pebble-floor test described by Rogers (1990). This test has been employed to study learning in chickens, as it involves a form of visual discrimination learning based on the chickens' ability to distinguish pebbles as a different 'food' from grains (Smith and Medin, 1981). The original protocol for the pebble-floor test requires chickens to search for grains on a background of pebbles, however, during preliminary testing we found the chickens not to be motivated to peck for the grains. Thus, the protocol was adapted to include (dead, but fresh) mealworms (FOOD), on a background of clay mealworms (DECOY). A square testing arena of 1.2×1.2 m (L x W), made out of plexiglass was used for all trials (Figure 3). The plexiglass floor was divided into 100 even squares, each containing 4 clay mealworms glued to the floor (n DECOY = 400). For each test, 200 mealworms were distributed evenly across the arena (2 mealworms per square, n FOOD = 200). The walls of the arena were 0.5 m high to prevent the chickens from fleeing the arena. All chickens were deprived of food (but not water) 3 h before testing. The order of testing was randomized on an individual level. Each chicken was allowed a total of 60 pecks at FOOD or DECOY. Pecks were recorded live using AutoHotkey in Excel on a laptop. Only pecks to new choices were scored, not repeated pecks at the same target (see Rogers, 1990; Rogers, 2007). If the chicken did not peck for 10 min after the start of the experiment, the test was terminated for that chicken. If the chicken started the test but stopped pecking for 5 min, the test was also terminated. For modelling the probability of completing the visual discrimination test, all 171 hens were included in the

Figure 3. Visual discrimination test. Left panel - testing arena with decoy mealworms. Right panel - chicken pecking at food (real mealworm) in the testing arena.

statistical analysis. In modelling the success rate, results of some hens were discarded (see "statistical analysis") leaving a total of 145 hens.

The chickens were tested binocularly (both eyes open), which is not the classic set-up of the pebble-floor test (see Rogers, 1990; Rogers, 2007). In several studies, birds are also tested monocularly, using 1 of 2 strategies: injection of glutamate into the left hemisphere of part of the animals, for example, Rogers and Bolden (1991) or covering the eyes with tape or a patch (e.g. Andrew et al., 2004; Dharmaretnam and Rogers, 2005; Wichman et al., 2009). As injections with glutamate were not possible/wanted for several reasons (animal welfare, needed to happen a couple of days posthatching, and would have interfered with other tests), covering one of the eyes was tried in a preliminary test. However, several strategies including paper patches, bandages, and children's socks with eyeholes all failed, and led to abnormal behaviors, such as freezing, slowly walking backward or scratching, and a cessation of all pecking behavior. The reason for these abnormal behaviors is unknown, as – to the best of our knowledge – previous pebble-floor testing has only been done in chicks, not in adult hens. To ensure the welfare of the animals, we opted to only do the test binocularly.

Hens with a better development of the left hemisphere (by light entering through the right eye during incubation, and hence brain lateralization) were expected to be more accurate in binocularly distinguishing between FOOD and DECOY. In chickens and pigeons, the left hemisphere controls pecking, enabling faster and more accurate responses (Güntürkün, 1985; Güntürkün and Kesch, 1987; Skiba et al., 2002) or inhibiting inappropriate responses (Deng and Rogers, 1997; Rogers, 2007). In addition, for chickens, it has been established that nearby objects (20–30 cm) are viewed using the binocular field rather than the monocular, which is used for more distant objects (Dawkins, 2002).

Statistical Analysis

In analogy with the pebble-floor test by Rogers et al. (1974) and Rogers (1990), the trial data for each hen were divided into blocks of 20 pecks. As such, if a trial was fully completed by a hen, this resulted in 3 blocks (**B1, B2, B3**) of 20 pecks (to either FOOD or DECOY). For all 171 hens the probability of completing the visual discrimination trial was modelled (see further). However, for the statistical analysis of success rate – the probability for each peck being a "success" or FOOD peck and not a 'failure' or DECOY peck – hens needed to perform at least 30 out of 60 pecks (so completed B1 and at least 10 pecks in B2), leading to a dataset of n = 145. Data were analyzed in RStudio (version 2023.09.1) using a binomial regression model with fixed factors including incubation treatment (Inc; light vs. dark, Inc+ vs. Inc-), and larvae rearing treatment (Lv; with vs. without, Lv+ vs Lv-). Whether or not the hens received range enrichment at the time of testing (EnR; with vs. without, EnR+ vs. EnR-) was added as a possible covariate (fixed factor). Possible interaction effects between fixed factors were tested. 'Pen' (i.e. housing pen during the rearing phase; 1-24) was added to the model as a random factor. "Laying group" (1–4) was tested as a random variable, but added zero variance to the model, and as such was not included. This model was employed to evaluate the probability of completing the entire visual discrimination trial (n = 171) and the overall success rate within the trial (n = 145). Starting from the most complex model, nonsignificant fixed factors were systematically removed in a stepwise fashion, resulting in a final model that exclusively retained variables with significant effects. To look into a possible effect of within-trial learning, the effect of block and the interaction with Inc, Lv, EnR on the success rate were tested in separate binomial regression models (n = 435).

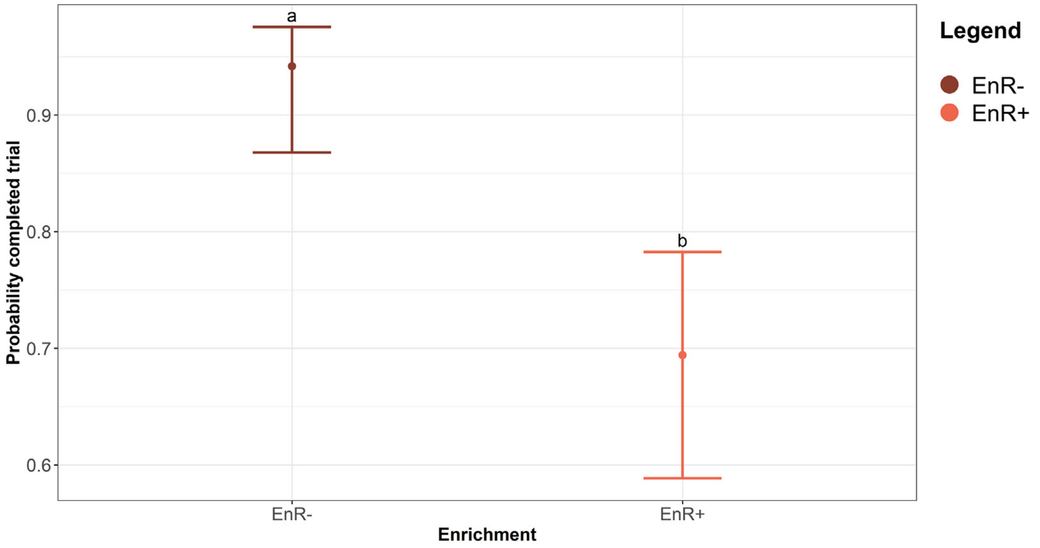

Figure 4. Effect of range enrichment on probability of completing the entire trial. LsMeans completed trials per enrichment treatment (EnR-: no range enrichment at the time of testing, EnR+: range enrichment at the time of testing) (different letters indicate significantly different groups [$P < 0.05$, n = 171]).

Effects with a P-value <0.05 were considered significant, effect size is reported through the odds ratio (**OR**) and associated standard error (**SE**). In addition, LsMeans and SE are also reported.

RESULTS

Effect of Incubation Treatment, Rearing Treatment, and Range Enrichment on Completed Trials

Only EnR had a significant effect of the probability of completing the entire trial (OR = −1.97 ± 0.52, z = −3.80, P < 0.001), with EnR+ having a lower probability of completing the trial compared to EnR- (0.69 vs. 0.94, Figure 4). In other words, chickens that received range enrichment at the time of testing were less likely to complete the visual discrimination trial. Inc and Lv had no significant effect on the probability of completing the entire trial (OR = 0.15 ± 0.42, z = 0.35, P = 0.727; OR = −0.16 ± 0.42, z = −0.38, P = 0.706, respectively).

Effect of Incubation Treatment, Rearing Treatment, and Range Enrichment on Success Rate

EnR was found to have a significant effect on the success rate (the probability for each peck being a "success" or FOOD peck) of the overall trial (OR = 0.30 ± 0.09, z = 3.58, P < 0.001, respectively). EnR- had a success rate of 0.927 ± 0.005, compared to 0.946 ± 0.005 for EnR+ (Figure 5). In other words, the success rate in the overall trial was higher for groups that received range enrichment at the time of the visual discrimination test. Inc and Lv were not significant with OR = −0.15 ± 0.13, z = −1.25, P = 0.213 and OR = 0.13 ± 0.13, z = 1.06, P = 0.29, respectively.

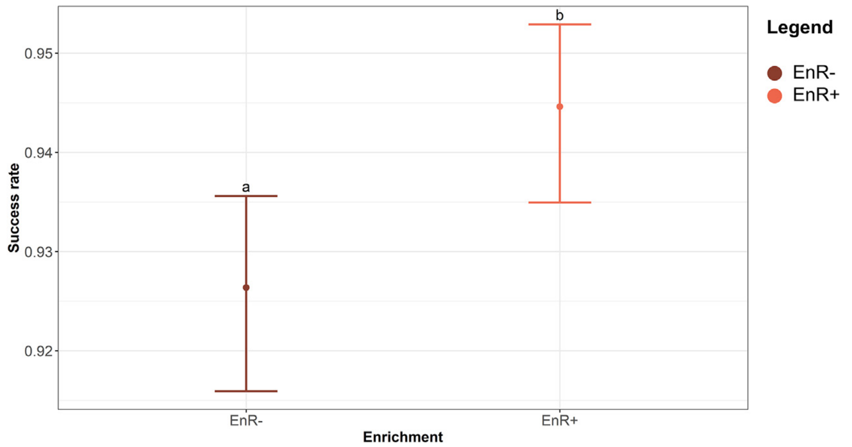

Figure 5. Effect of range enrichment on success rate in the overall trial. LsMeans of success rate in overall trial per enrichment treatment (EnR-: no range enrichment at the time of testing, EnR+: range enrichment at the time of testing) (different letters indicate significantly different groups [$P < 0.05$, n = 145]).

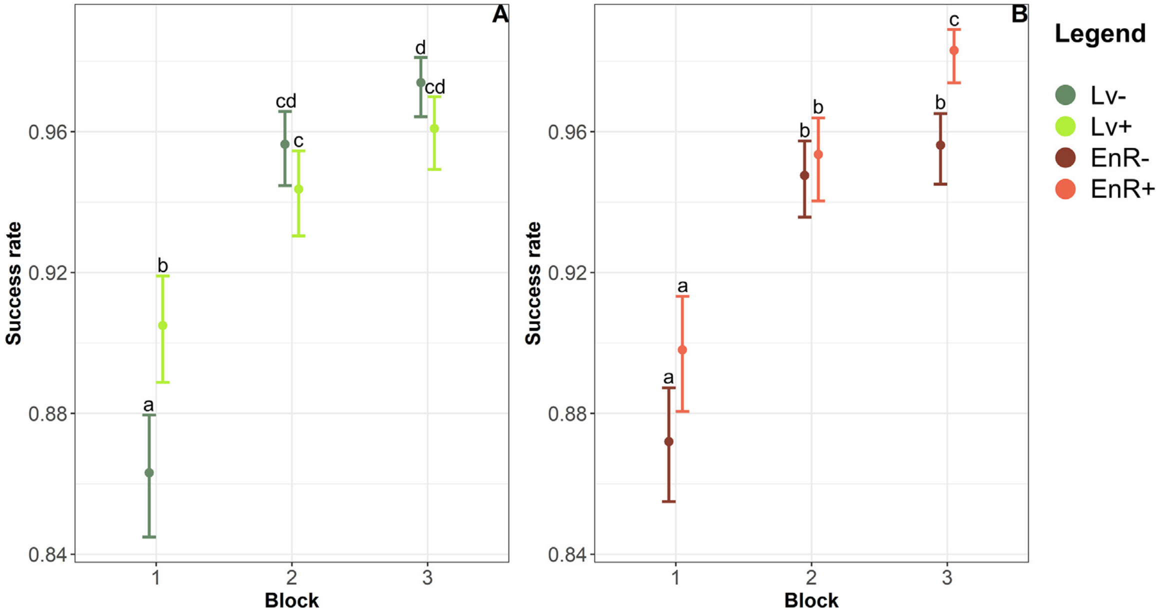

Figure 6. Effect of rearing treatment and range enrichment by block on success rate. A. LsMeans of success rate in overall trial per block per rearing treatment (Lv-: rearing without BSFL; Lv+: rearing with BSFL). B. LsMeans of success rate in overall trial per block per enrichment treatment (EnR-: no range enrichment at the time of testing, EnR+: range enrichment at the time of testing) (different letters indicate significantly different groups [$P < 0.05$, n = 435]).

Effect of Incubation Treatment, Rearing Treatment and Range Enrichment on Success Rate By Block

No significant interaction effect was found between block and Inc on success rate (OR = −0.21 ± 0.12, z = −1.72, P = 0.086). For Lv and EnR a significant interaction with block was present (OR = −0.50 ± 0.12, z = −4.13, $P < 0.001$ and OR = 0.27 ± 0.13, z = 2.09, P = 0.036, respectively, Figure 6). In other words, incubation treatment did not affect within-trial learning, whereas BSFL rearing, and on-range mealworm enrichment did.

Lv- and Lv+ differed from each other only in B1 (OR = 1.51 ± 0.18, z = 3.54, P = 0.006). For Lv-, B1 was significantly different from B2 (OR = 3.48 ± 0.52, z = 8.42, $P < 0.001$) and B3 (OR = 5.93 ± 1.08, z = 9.75, $P < 0.001$). For Lv+, B1 was also significantly different from B2 (OR = 1.76 ± 0.26, z = 3.87, P = 0.0015) and B3 (OR = 2.58 ± 0.43, z = 5.74, $P < 0.001$). B2 and B3 were not different for both Lv- and Lv+. In other words, both groups exhibited within-trial learning between B1 and B2. However, Lv+ had a significantly higher success rate than Lv- at the start of the trial.

EnR- and EnR+ differed from each other only in B3 (OR = 2.66 ± 0.68, z = 3.82, P = 0.002). For EnR- B1 was significantly different from B2 (OR = 2.65 ± 0.35, z = 7.329, $P < 0.001$) and B3 (OR = 3.20 ± 0.46, z = 8.19, $P < 0.001$), with no difference between B2 and B3. For EnR+ all blocks were significantly different: B1-B2; OR = 2.33 ± 0.38, z = 5.21, $P < 0.001$; B1-B3; OR = 6.59 ± 1.60, z = 7.75, $P < 0.001$ and B2-B3: OR = 2.83 ± 0.74, z = 3.96, P = 0.001). In other words, both groups exhibited within-trial learning, though an extended learning process was visible in EnR+.

DISCUSSION

The present study investigated the effects of light during incubation (**Inc**) and rearing with BSFL (Lv) on the visual discrimination abilities in laying hens. Due to an ongoing experiment on range use, half of the hens also received mealworm enrichment (**EnR**) at the time of the visual discrimination trial. In this discussion, all treatments are first considered separately, with extra attention to the results by Manet et al. (2023b), Kliphuis et al. (2023) and Kliphuis et al. (2024), as their tests involved the same individuals, in earlier life stages. The final part of the discussion is devoted to the testing conditions and some wider implications of the results found in our study.

Effect of Incubation

We found no evidence of an effect of light during incubation on the visual discrimination abilities of the hens. It is important to note that we cannot conclude that there was no effect on brain lateralization through light during incubation. We can only conclude there was no effect on the categorization of food and nonfood items as tested in our experiment. The visual pathway is only one of the aspects that can be affected by brain lateralization (see Rogers, 2007 for a comprehensive overview), and within the visual skills, discrimination or categorization abilities are just 1 facet. Apart from the testing conditions (see further), 2 other reasons could have contributed to a lack of effect of light during incubation on the success rate within the visual discrimination test: incubation conditions, and chicken characteristics.

It is worth considering that the incubation conditions used in our set-up might not be optimized for full effect.

Light color and intensity, but also the timing during incubation as well as the photoperiod (hours per day) could all have an effect. With regard to the latter, our study employed a photoperiod of 12L/12D during the full 21 d of incubation, similar to Özkan et al. (2022) (who employed 16L/8D during 21 d). Another way to go would be to better mimic natural conditions, where only during the very last days of incubation short periods of light would reach the embryo. Rogers (1996) stated that 2 h of light per day on incubation days E19-20 would be enough to establish lateralization of the visual pathway in domesticated broiler chickens. Archer and Mench (2014b) looked into the 'natural incubation pattern in a commercial setting', meaning that mother hens were allowed their natural brooding pattern, but—as is the case in a commercial setting—feed and water were readily available (so not much foraging effort was needed). Their test revealed that these hens would spend even less time away from their eggs, resulting in an even shorter photoperiod than the 20 min mentioned by Mrosovsky and Sherry (1980) or the 2 h by Rogers (1996). While hens indeed spent most of the time on the nest, they left more frequently during the last week, though for shorter periods of time, with a mean time of only 4 min/d. If these are indeed the more natural conditions, the photoperiod in our and most other studies was excessively long. With regard to timing, Archer and Mench (2014b) showed that providing light only during the sensitive period of visual development generates chicks very similar in characteristics to dark-incubated chicks, while chicks that were light-incubated during the full incubation period showed a difference in lateralization. This aligns with growing evidence in zebrafish (Budaev and Andrew, 2009) and chicks (Rogers et al., 2013) that light stimulation before the sensitive period of visual development affects lateralization. Results with regard to light color have been inconsistent, with Özkan et al. (2022) reporting positive effects for incubation with green light, while Archer and Mench (2017) found very little effect of green light (and a positive effect for white and red light). Overall, a great deal of uncertainty still exists with regard to an optimized light regime during incubation, so further research is recommended.

Furthermore, it is possible that the effect of light during incubation on lateralization in our (female) brown laying hens was less pronounced (and hence difficult to detect). The strongest effects of light during incubation on lateralization have been demonstrated in male broilers (e.g. Rogers, 1996). The level of circulating sex hormones − namely a drop in plasma testosterone in males − likely causes a sex difference in the degree of brain asymmetry between males and females, with males being more sensitive to the effects of light stimulation (Adret and Rogers, 1989; Rajendra and Rogers, 1993; Rogers, 1996; Rogers, 1993; Tanabe et al., 1979; Woods et al., 1975). In addition, considering the fact that light transmission through the eggshell has been found to be lower in brown eggs compared to white eggs (Manet et al., 2023b), it is possible that lateralization of the visual pathway is less pronounced in brown hens. Though the effect of light during incubation was found to be similar with regard to hatching characteristics (Manet et al., 2023b) and fear responses (Manet et al., 2023a) in white and brown chicks, differential effects during the laying stage have not been investigated yet (to the best of our knowledge).

It is also important to note that we found no negative effect of light during incubation on the visual discrimination abilities. Therefore, if light during incubation has a positive effect on other stages (hatching or pullet) or on other characteristics (e.g. feather pecking or fearfulness) in adult hens, it is still worth further investigation. Results in that regard have been inconsistent, with some studies reporting a reduction in feather pecking (pullets and layers, Özkan et al., 2022) or fearfulness (broiler chicks, Archer, 2017; Archer and Mench, 2014b; Archer and Mench, 2017), and others an increase in (gentle) feather pecking (layer chicks, Riedstra and Groothuis (2004) or fearfulness (layer chicks, Dimond, 1968). Manet et al. (2023a) only found a positive effect of light during incubation on fearfulness in 1 out of 15 behavioral tests in the chick/pullet stages (5 and 10 wk). Kliphuis et al. (2023) found no effect of light during incubation on fearfulness on group level, feather pecking, plumage condition, foraging behavior or behavioral recovery time after vaccination, and Kliphuis et al. (2024) reported a small positive effect of light during incubation on fear of humans in individually tested pullets.

Effect of Rearing With BSFL

Interestingly, hens that were reared with BSFL were more successful in the first block of the visual discrimination trial compared to hens that were not reared with BSFL, indicating that BSFL-reared hens had a head start. However, in the second block, non BSFL-reared birds had already caught up, suggesting quick learning once an interesting enrichment is available. This demonstrates once more the value of enrichment at any life stage of the bird. In provisioning BSFL during rearing, Kliphuis et al. (2023) already discovered an effect on foraging behavior. Though total foraging duration was not affected, BSFL-enriched pullets did initiate more foraging bouts. These results might imply that the provisioning of larvae during rearing caused more frequent, but shorter, efficient moments of foraging. These results are similar to results by Blokhuis and Van der Haar (1992), who found that hens reared with grains, showed a more efficient foraging behavior in the pullet stage (less exploratory pecking). In that study, some differences with regard to pecking were also observed in the laying stage, suggesting that rearing with grain had changed pecking behavior in a more permanent way.

Assuming rearing with BSFL indeed has a positive effect on the visual discrimination abilities in later life, it is also important to look at the bigger picture, *i.e.* are there any known negative effects of BSFL during rearing? Aside from the previously mentioned effect on foraging (Kliphuis et al., 2023), studies have shown either no effects or beneficial effects of using live BSFL enrichment during rearing. In summary, no effect of live BSFL was found on feed conversion ratio, egg parameters (i.e. egg production, egg weight, shell strength, shell breaking strength or Haugh unit) (Star et al., 2020; Tahamtani et al., 2021), fearfulness (Kliphuis et al., 2023; Kliphuis et al., 2024; Tahamtani et al., 2021), feather pecking (Kliphuis et al., 2023), behavioral recovery time after vaccination (Kliphuis et al., 2023), or corticosterone levels in plasma and feathers (Kliphuis et al., 2024). A desirable effect of BSFL was found on the facilitation of expression of natural behavior, plumage condition and feather pecking (Star et al., 2020), as well as weight gain (Tahamtani et al., 2021). However, for the latter 3 parameters, results are inconsistent with Kliphuis et al. (2023) not finding an effect of BSFL. Overall, not taking insect welfare into account (as this is beyond the scope of this paper), BSFL remain a good candidate for (early-life) enrichment.

The impact of rearing conditions on the laying phase has been reviewed by Janczak and Riber (2015), recognizing the long-term effect of different management strategies and environmental conditions. Several studies have stressed the importance of an optimized rearing period, particularly for birds destined for more complex housing systems (Colson et al., 2008; Häne et al., 2000; Leenstra et al., 2014; Staack et al., 2007). Janczak and Riber (2015) also stated that − to achieve optimal welfare and even productivity of layers − it is best to match the rearing housing system with the layer housing system. Though our study did not look into housing, likely the same principle can be applied to foraging, i.e. if hens were raised with live larvae, they might be better suited or equipped for insect foraging in an outdoor environment. Campbell et al. (2019) stated that visual stimulation might be particularly beneficial for birds that are destined for free-range production.

Effect of range Enrichment at the Time of Testing

Though the goal of this study was to investigate long-term effects of early-life measures and not enrichment during the laying period, this still had to be taken into account (as half of the hens received mealworm enrichment for a study on range use). Birds that received mealworm enrichment at the time of testing were less likely to complete the entire trial, compared to those that received mealworms at an earlier stage. This might be related to a degree of habituation, for chickens that had mealworms at their disposal during the testing period, the attractiveness or novelty of the mealworms might have been reduced during the visual discrimination trial itself. Indeed, Tahamtani et al. (2021), in comparing *ad libitum* larvae with diets containing 10% and 20% larvae, found that the hens would eat less voraciously in the *ad libitum* situation. Instead of consuming the entire portion of larvae within 5 min, they would space out consumption across the day. The authors proposed this is due to a decrease in the viewed value of larvae as they were always available. Though our provision of mealworms was not *ad libitum*, the 4 kg of mealworms provided lasted the hens on average about 2 to 2.5 d. This implies, though they were very eager to eat the mealworms (personal observation), the hens did somewhat space out their consumption over a few days. For the chickens that did reach the criteria of trial completion, range enriched hens were more successful during the trial compared to hens that did not receive mealworms at the time of testing. These findings align with the results by Blokhuis and Van der Haar (1992), who found that supplying grain as enrichment changed the character of food pecking, making it less exploratory (intended rather to gather information about food and not primarily to eat it) and hence more efficient.

Testing Conditions

Though visual discrimination abilities have traditionally been tested with the pebble-floor test, it has only been performed in chicks, not adults (e.g. Chiandetti and Vallortigara, 2019; Rogers et al., 2007; Rogers, 1997). This makes for a hard comparison, as we have no indication how suitable this test actually is for adult chickens. Us failing to test the adult hens monocularly and only binocularly might also be a limitation of this study. This difficulty in testing may well be one of the reasons why so little is known about the effect of light during incubation on lateralization in adult chickens. Rogers (1996) stated that the biggest effect of light during incubation on lateralization of the visual pathway is visible in the first 2 to 3 wk of age (in broiler chicks), but whether these effects persist until adulthood is unknown. Further studies on the presence and degree of lateralization in the laying stage are warranted.

In addition, though all groups had a certain degree of experience with mealworm-shaped enrichment (due to the crossback-crossover design of the range enrichment during the laying phase), BSFL-reared hens had substantially more experience with mealworm-shaped enrichment (every d from 1 to 19 wk of age), compared to non-BSFL-reared hens. Due to the similar appearance of BSFL and mealworms, it cannot be completely excluded that this previous experience made BFSL-reared hens more adept in simply discriminating between mealworms and decoy mealworms (or other mealworm-shaped foodstuffs), instead of having better visual discriminating abilities overall. A solution might be a repetition of the visual discrimination trial with fruit or grain items, though this poses the risk of hens not being motivated enough to complete the test (see materials and methods).

Wider Implications

We found no evidence of lateralization of the visual system through light during incubation in adult laying hens.

But why would lateralization be a useful trait in animals, and could domestication have had an effect on the degree of lateralization in laying hens? On an individual level, lateralization can increase neural capacity, because functions can be divided between hemispheres (Levy, 1977), see also the well-known dual task experiment by Rogers (2000). However, side biases in perception or behavior might also be disadvantageous, because predators, food or even social companions might appear both on the right or the left side of the animal. It has been proposed that lateralization actually occurs on a population level, rather than the individual level. The aligning of the direction of behavioral asymmetries at the population level could have arisen as an 'evolutionarily stable strategy' that occurs when asymmetrical individuals need to coordinate their behavior with other asymmetrical individuals (same species or different species, e.g. a predator) (Vallortigara and Rogers, 2005). However, so far this is just a theory when it comes to avian species (or even vertebrates), as the evidence thereof only exists in highly social honeybees (Anfora et al., 2010).

The possible lack of lateralization in adult laying hens might also be part of what is called 'the domestic phenotype'. In a wide range of animals, domestication has brought with it changes in appearance and behavior (some even very similar across species such as changes in coat/plumage color) (Trut, 1999). The transition from wild (free ranging) to captive status is inherently accompanied by changes in the availability and/or accessibility of shelter, space, food/feed and water, as well as less predation and a different social environment. Through (genetic) mechanisms such as inbreeding, genetic drift, artificial selection, natural selection in captivity and relaxed selection, domestic phenotypes can occur over time. The role of the environment and associated experiences, such as the presence or absence of key stimuli, changes in intraspecific aggressions and human interactions as well as humans acting as a buffer between the animal and its (natural environment) are key. As such, domestication has resulted in modified rates of behavioral and physical development (Price, 1999). All of this begs the question whether a lateralized brain (or visual system) is still part of the laying hens' domestic phenotype. As the domestication process has—in many cases—reduced the sensitivity to their environment, a strong degree of lateralization has perhaps lost its use in a domesticated or commercial setting. It would be interesting to investigate whether the extent (or duration with age) of lateralization has diminished in domesticated chickens, compared to more wild type chickens. In addition, it could be argued that − though lateralization serves perhaps little purpose in a conventional farm setting − it can be useful in a more natural setting, where there is still a predation risk while the animals are foraging. If there is indeed less lateralization in the domestic phenotype of chickens, for outdoor production it might be worth considering breeds that are closer to wild type chickens to ensure animal welfare. Of course, for now this is just a conjecture, further investigation is necessary to confirm this theory.

The results from our study highlight the importance of enrichment during rearing, with BSFL having a positive effect on the visual discrimination abilities of adult laying hens in the first block of the visual discrimination trial. This is in accordance with other studies stating that the adaptation to a more complex foraging environment during rearing is essential for later thriving in an outdoor environment (Campbell et al., 2018; Janczak and Riber, 2015). Of course, both in a conventional and outdoor setting, feed is readily available for the birds, but better visual discrimination skills would seem beneficial in an outdoor environment, as they might lead to more efficient foraging behavior outside (e.g. insects), which may in turn even affect range use and feather pecking (and ultimately the welfare of the birds). The effect of BSFL during rearing on feather pecking and range use will be discussed in 2 other papers (Plante-Ajah, in progress).

CONCLUSIONS

Incubation with green light did not have an effect on the visual discrimination abilities of adult laying hens, as tested in a modified pebble-floor test. Rearing with BSFL did have a positive effect on the visual discrimination abilities (though only during the first block of the trial), which might be useful in a more complex free-range setting where the animals have more foraging opportunities. Range enrichment at the time of testing led to a lower degree of test completion, likely due to habituation to the mealworms as an enrichment. However, from the birds that completed the test, hens that had range enrichment at the time of testing, had a higher success rate compared to those who had range enrichment at an earlier stage. The positive effects of BSFL during rearing and mealworms during the laying period stress the importance of enrichment throughout the life of the hens.

ACKNOWLEDGMENTS

This study was part of the PPILOW project (Poultry and Pig Low-Input and Organic production systems' Welfare) and the ChickenStress European Training Network. PPILOW has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°816172. ChickenStress received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement N°812777. The authors would like to thank Dimitri Van Grembergen, Dana De Wart, Evelien Graat, Anja Van Havermaet and Marleen van Yperen for assistance with sampling and the poultry caretakers at ILVO.

DISCLOSURES

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.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.104236.

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