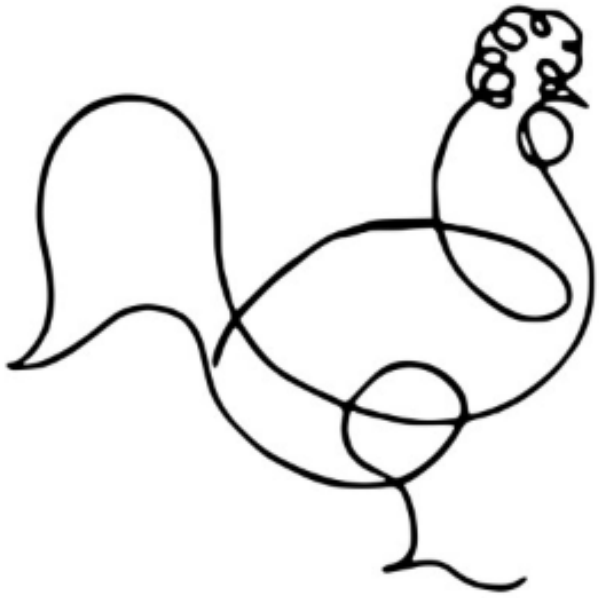

The effects of early life treatments on stress responsivity in laying hens during rearing

Master thesis

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Introduction

Feather pecking (FP) and cannibalism among laying hens is a threat to animal welfare and, furthermore, has serious economic consequences. Feather pecking can be either gentle or severe (Rodenburg et al., 2013). Gentle feather pecking is a result of social hierarchy and exploration (de Haas & van der Eijk, 2019). Severe feather pecking causes serious damage in commercial non-caged flocks. It is considered a pathological behaviour, and often the result of suboptimal conditions and can be a stress coping mechanism. Star et al. (2020) state that a lack of social hierarchy, due to modern non-cage systems, enhances feather pecking. Many factors influence feather pecking behaviour, such as rearing conditions, type of breed, feeding strategies, housing conditions but also maternal stress or incubation conditions (Van de Weerd & Nelson, 2006; Rodenburg et al., 2013). One of such incubation conditions, namely the effect of exposure to light, reduces stress and improves the immune system (Özkan et al., 2012; Archer & Mench, 2014). These studies were performed on broiler chicks. Nonetheless, this possibly reduces feather pecking, fearfulness and fear sensitivity post-hatching for laying hens as well.

In this study we will investigate innovative and biological relevant solutions against feather pecking, fearfulness, stress coping mechanisms and their effect on behavioural development. By biological we mean solutions based on species-specific behaviours or environmental reactivity, as opposed to fabricated or artificial, such as hormones or medication.

Cerebral lateralisation

Cerebral lateralisation is observed in many vertebrates, also in chickens (Vallotiraga et al., 1999). Laterality is prevalence of the left or right hemisphere of the brain when performing everyday tasks in life. These tasks include foraging, social interactions, but also agonistic responses (Vallotiraga & Rogers, 2005). The left brain hemisphere plays an important role during visual tasks, sound production, foraging behaviour and social recognition. On the other hand, the right hemisphere is specialised in agonistic responses (Archer & Mench, 2017). To put it differently, the left hemisphere is more involved in routine behaviour, whereas the right hemisphere is more involved in novel stimuli (Rogers, 2010).

Laterality of the brain may enhance survival, as the brain works more efficiently during decision-making. Exposure to stimuli and a complex environment in early life, enhances a lateralised brain. According to Vallotiraga & Rogers (2005), such a brain, "has increased functional capacity" (p. 621). Little is known about the development of laterality of visual functioning during incubation. Evidence suggests that chicks can respond to environmental stimuli prior to hatching (Lauber, 1974; Reed & Clark, 2011;). Such environmental stimuli can be olfactory, auditory but also photoperiodic cues.

Light stimulation has its effect the last days prior to hatching (Vallotiraga & Rogers, 2005). During three to four days prior to hatching, the chick is susceptible to light, but the chick is positioned in such a way that the right eye is exposed to light, whereas the left eye remains positioned under the wing, and is therefore not exposed to light (Rogers & Krebs, 1996). This unilateral exposure to light founds the lateralization of visual projections and visual functioning in the chick (Rogers & Krebs, 1996). Moreover, eggs that are incubated in the dark during the final days before hatching, do not develop such asymmetry in visual projections (Andrew et al., 2004).

It is already known that exposure to light prior to hatching develops asymmetry of visual projections in the brain, which consequently affects visual functioning, resulting in different behaviour depending on usage of the right or left eye. Using the asymmetric functioning of its visual system, a chicken interacts differently with its environment. This also affects its capability of functioning in a social group (Daisly et al., 2009). Moreover, it has been shown that fear response is also lateralised (Philip & Youngren, 1986). It appears that the right hemisphere is involved in fear responses in chickens, and that there is a more efficient response to predators when they are detected with the left eye opposed to detection with the right eye (Rogers et al., 2004). The hemisphere and the eyes are positioned ipsilaterally. In other words, if the right hemisphere is controlling in a fear response, the chick will use its left eye more to process information from its environment (Vallortiraga et al., 1999). All in all, it is interesting to see whether a regular light-dark cycle affects development of the brain in terms of lateralisation, and therefore affects social behaviour, fearfulness and feather pecking.

Early life enrichments

Furthermore, environmental enrichment in early life also influences stress coping mechanisms. Environmental enrichment, such as providing foraging material or food enrichment, promotes foraging behaviour. Foraging behaviour is natural behaviour for chickens, and according to Star et al (2020), free-range chickens even spend up to 37% of their time foraging for live insects. Accordingly, if they are not able to express their natural behaviour, this may result in increased aggressiveness and feather pecking (Star et al., 2020). Consequently, promoting foraging behaviour decreases damaging behaviour and, therefore, feather pecking (Aernie et al., 2000). Moreover, providing nutritious early life enrichment may also positively affect health, such as feather condition and egg quality (Star et al., 2020). As a result, this improves production performance (Steenfeld et al., 2007). Presenting chickens with live insects, such as larvae of the black soldier fly (*Hermetia illucens),* are a nutritious and sustainable form of life enrichment (Star et al., 2020). ISA brown laying hens are commonly used in the laying industry. They are known for their docile character, but also show feather pecking.

Behaviour tests & hypotheses

We measured the effects of light-dark cycle and food enrichment on damaging behaviour, stress coping mechanisms and behavioural development. The lateralisation test and a behavioural recovery test measure these effects. These tests measure fear coping behaviour as well as social behaviour, such as feather pecking. The lateralisation test measures a chick's preferential eye use, by having them cross a barrier either on the right or on the left. Furthermore, it measures latency to choose a side and latency to vocalise. If early life treatments positively affect social development and stress coping mechanisms, the group that was incubated under light-dark circumstances (LDI group) will show different behaviour compared to the group that was incubated under standard dark circumstances (DI group). For example, the latency to vocalise will be shorter in the DI group as increased distress vocalisation can be a sign of stress response (Feltenstein et al., 2002; Marx et al., 2001). The latency to emerge and cross a barrier will probably be shorter with chickens that have better stress-coping mechanisms, because they are less fearful, and the chicks that have right-hemisphere dominance will tend to pass the barrier on the left.

During the behavioural recovery test, we were interested in recovery back to baseline behaviour after a stressor, in this case a vaccination, injected in the wing, an eye droplet and a breast injection, applied consecutively. We score a time-budget with a scan sample of the home pen during the day, before the stressor (baseline) and after the stressor (vaccination). We score behaviour, such as dust bathing and feeding, but also feather pecking and alertness (see fig. 2). If the chicks that received early life treatments have better stress coping mechanisms, they will be better able to cope with a stressor, such as vaccination, compared to the group that did not receive treatment. For that reason, we expect that the birds in the LDI group will recover more quickly from a stressor compared to the DI group, and will, therefore, return more quickly to baseline behaviour.

All in all, during these tests, we expect to see a difference between the groups that received early life treatments compared to the group without early life treatments. Hence, our hypothesis is that we expect to see a difference in feather pecking behaviour, fearfulness and stress sensitivity between the LDI and DI groups as a consequence of early life interventions. Furthermore, we expect to see a difference in feather pecking behaviour, fearfulness and stress sensitivity between the larvae and no-larvae group as a consequence of early-life enrichment.

Material and methods

Ethical note

This project was approved by the Master Onderwijs Commissie (MOC) at Utrecht University, the Centrale Commissie voor Dierproeven (CCD), the Instantie voor Dierenwelzijn (IvD), and the Dierexperimenten Commissie (DEC).

Birds and housing

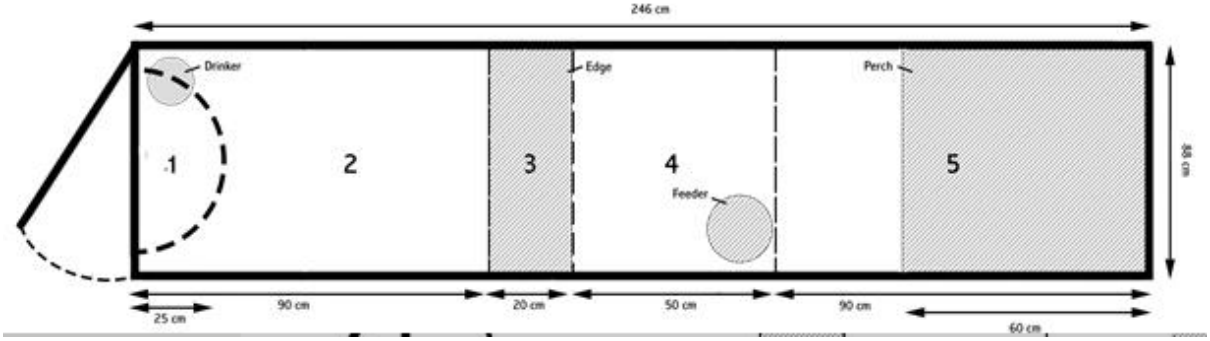
500 Eggs of ISA brown were incubated in a total of four HatchTech incubators. Two groups were incubated under standard dark conditions, whereas the other two groups were incubated under L:D=12:12 with green light (Archer & Mench, 2014). A total of 200 of ISA Brown hens were used during this study. The male chicks (approximately 50% of the chicks) were excluded from the study and humanely euthanised after hatching. Three female chicks were excluded from the study: two chicks did not fully hatch from the egg, and the third

chick was euthanised because it had reached a humane endpoint. The humane endpoints are defined as follows: "*If the adult birds lose 10% of their body weight and are expected to be suffering upon inspection, they will be removed from the experiment. This will also be the case if other signs of severely impaired health are observed, for example, when chicks are huddled down, show a strongly reduced mobility and are in poor condition. When wounds due to feather pecking or cannibalism arise on the skin or toes, we will treat birds with disinfecting CTC spray and repellent PBH spray twice. When there is no improvement after the treatment and birds are still targeted by feather peckers, animals will be removed from the experiment. However, we do not expect these clinical signs as a consequence of the procedure*".

Additionally, five male chicks had to be euthanised because of incorrect sexing after hatching. After hatching, 197 female chicks in groups of 9, 10 or 11 were randomly placed in 20 246cm x 88cm pens (see fig. 1), comparable to free range and organic farming systems with natural light. Food and water are given ad libitum.

During the rearing phase (0-18 weeks), two groups were compared: one group was incubated with a regular (12:12) light-dark cycle (LDI group) and the second group was incubated under standard dark conditions (DI group). Half of the birds of each group were provided with black soldier fly larvae (*Hermetica ilucens)* in a larvae feeding tube. These feeding tubes are 15 cm long, 4 cm wide and have 3 holes each (see fig. 4). The larvae tubes worked as food puzzles, as the chicks had to find out how to get to the live larvae through the holes (foraging behaviour). In short, there are four groups: LDI/larvae, LDI/no larvae, DI/larvae and DI/no larvae. The four conditions (LDI/larvae; LDI/no larvae; DI/larvae; DI/no larvae) were not mixed within a pen.

Figure 1
Schematic view of the home pens. 1= 2= 3= 4= 5=perch



Behaviour tests & ethogram
The two following tests were conducted on the hens during the rearing phase:

1) Behavioural recovery test

During the day, after a three-fold consecutive wing web vaccination, eye drop vaccination and an intramuscular breast vaccination, we conducted a scan-sample of the home-pen. Consequently, we scored their social and foraging behaviour, such as feeding, feather pecking or dust bathing (Ericsson et al., 2014, see fig. 1). Immediately after the last animal received vaccinations, we conducted a scan sample. During a 60-minute recovery phase, we returned to their pen every ten minutes, and conducted a scan sample. All in all, we made seven scan samples in six time -intervals (T0-T6).

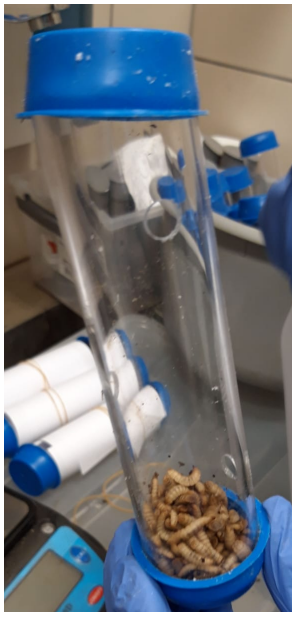
Additionally, after ten days the home-pen observation was repeated in the same manner, in order to obtain a baseline observation. By comparing baseline behaviour to behaviour after vaccination, we get an impression of stress-coping behaviour. Before starting the observation, we let the hens habituate to our presence by walking back and forth in front of their pens for fifteen minutes.

Figure 2
The ethogram with recorded behaviours measured including their description (Ericsson et al., 2014).

Behaviour	Description
Relax	Stand, sit or walk with reduced attention with no alert head movements and short neck. In standing and sitting, eyes may be partially closed
Preen	Grooming themself, with beak 'brushing' their feather.
forage larvae	When they forage the larvae tube
Forage	Pecking at the ground or the environment. Scratching with their feet.
Feed	Eating from yellow feeding tray.
Drink	Drinking from their drinking bucket.
Dust bath	Combined preening and scratching behaviour during which the chick pecks and scratches at the dust bath area, then squats down and follows an organized sequence of behaviour patterns such as head rubbing and vertical wing shaking.
Alert	Stand, sit or walk with eyes opened and raised neck, attendant to the surroundings but not to floor, feed- or water bucket
Feather ruffle	Erects feathers, ruffles, and shakes body
Wing flap	Flaps wings while standing on ground or perch
Severe feather peck	Pulling at feather of conspecific

Figure 3
Set-up of the lateralisation test apparatus. The chick was placed in front of a transparent plate and had to choose right (R) or left (L) to cross the plate.

Figure 4
The larvae tubes as they were used in this experiment. They contained live larvae and were situated on the floor of the pens



2a) Lateralisation test

Briefly, we put the chick in a square apparatus (see fig. 3), in front of a transparent plate. On the opposite wall, there is a mirror, so the chick can see this mirror and, therefore, itself. This serves as a social motivation to cross the barrier. Additionally, as an auditory motivation, we played a pre-recorded sound of their home pens from behind the mirror. All in all, we assume they had both a social as well as an auditory stimulus to cross the barrier.

We checked the latency to choose a side and the side chosen; we also counted the number and latency to vocalise, since this can be an indication of stress behaviour (Feltenstein et al., 2012; Marx et al., 2001). A total of 64 chicks were tested, because we simply ran out of time before we could test more chicks. We allowed a time limit of 300 seconds for the chick to detouring the transparent plate. Chicks that had not passed the plate by then, were given the maximum score of 300. We did six trials with each chick, then calculated a laterality index (LI) as follows: (detour to the right - detour to the left) / (detour to the right + detour to the left) x 100. The LI assesses lateral asymmetries in the direction of the detour. This gives us an insight in right or left hemisphere dominance. If exposure to light affects lateralisation of visual functioning, this affects the chick's ability to cope with its environment and thus affects social behaviour (Daisley et al., 2009).

2b) Lateralisation test: social reinstatement

The lateralisation test gives a variety of variables, such as the latency to emerge and the latency to cross a barrier, which provides data about social reinstatement. Therefore, within the construction of the lateralisation test, a social reinstatement test was conducted as well. This provided us insight in their stress-coping behaviour.

Statistics

Behavioural recovery test

Validation

One assumption of repeated measures ANOVA is that of sphericity. This is the condition where the variances between the repeated measures are equal. The Mauchly's test measures sphericity. If the significance in Mauchly's test is higher than 0.05, the sphericity can be assumed. If sphericity is significant, the assumption is violated. This can be corrected by a Greenhouse-Geisser-, Huynh-Feldt- & lower-bound correction, because they apply a correction factor to the F-values. According to Field (2009), when the Greenhouse-Geisser estimates an epsilon value more than 0.75, the Huyn-Feldt correction should be used. For all tests where sphericity was violated, the epsilon value exceeded 0.75, therefore, we chose to use the Huyn-Feldt correction for all the tests where sphericity could not be assumed.

In order to perform a repeated measures ANOVA, we had to remove time interval 0 for all pens. For time interval 0, there was missing data for pen 17. Consequently, data was unbalanced. This way, we could perform a repeated measures ANOVA.

1) Behavioural recovery test: repeated measures ANOVA

For every behaviour category, a proportion was calculated by dividing the count of behaviour by the total number of chickens in a pen. A baseline was calculated by taking an average proportion for every behaviour category in a time interval. In order to perform statistical analyses, a deviation from baseline score was calculated, similar to the method used by Ericsson et al (2014). Deviation from baseline is the proportion of behaviour on vaccination day in a certain time interval minus proportion from baseline. This outcome was called the deviation score. The deviation score was used as an outcome variable to evaluate how long it took the different treatment groups to recover back to baseline. We performed a repeated measures ANOVA on the deviation score on the four different treatment groups. Furthermore, we also performed a measures ANOVA on the deviation score for two treatment groups, with larvae and incubation as between-factors. The four treatment groups are not independent, but we did this because the sample sizes were small because of a randomisation error. For both repeated measures ANOVA a time effect was included. Time was used as a within-subjects factor, whereas treatment was used as a between-

subjects factor. For both tests, the differences were considered significant if *P*-values were below 0.05. When *P*-values were between 0.05 and 0.1, this was considered as a tendency.

Lateralisation test

Validation

Normality assumption was violated for all data; therefore, we transformed all data according to a log transformation. After that, we performed a repeated measures ANOVA because the test is said to be robust (Field, 2009), except for the LI, because this data was not a repeated-measures data and normality violation could therefore more easily be resolved by using a non-parametric test, namely a Kruskal-Wallis test (see table 1).

2a) Laterality index: Kruskal-Wallis test

A total of 64 chicks were tested in the Laterality Index (LI) test. After recording left (L) or right (R) for each chick after the six trials, we calculated a Laterality Index (LI). To evaluate whether treatment had effect on the LI, we performed a Kruskal-Wallis test on the data. This is a non-parametric equivalent of the one-way ANOVA, which is preferred when normality is violated (Field, 2009).

Treatment effect of Incubation: Kruskal-Wallis test

Since we expect the light-dark incubation to have an effect on the lateralisation of the brain, we also looked at the effect of incubation treatment separately, so without the effect of larvae treatment, by performing a Kruskal-Wallis test.

2b) Latency to vocalise: repeated measures ANOVA

For each trial we recorded the latency to vocalise. We scored the chick with a maximum score of 300 for latency to vocalise, if we recorded no vocalisations for that chick.
After a log transformation, we performed a repeated measures ANOVA latency to vocalise. Treatment was a between-groups factor. Trials were used as a within-groups factor.

2c) Number of vocalisations: repeated measures ANOVA

For each trial we recorded the number of vocalisations. If we recorded no vocalisations, the chick was scored with a minimum score of 0. We performed a repeated measures ANOVA for number of vocalisations, after a log transformation. Trials were used as a within-groups factor, and treatment as a between-groups factor.

2d) Social reinstatement. Latency to pass barrier: repeated measures ANOVA.

For each trial we scored the chick with the time it took to pass the barrier, with a maximum score of 300 s. After a log transformation, we performed a repeated measures ANOVA on data, with trials as a within-groups factor, and the four treatment groups as a between-groups factor.

Table 1
Summary of the statistic tests that have been used for the lateralisation test

Parameter within Lateralisation test	Data transformation	Test	Within-groups factor	Between-groups factor	Significance level
Laterality Index (LI)	none	Kruskal-Wallis	-	-	P<0.05
Latency to vocalise	Log transformation	Repeated measures ANOVA	trials	Treatment groups	P<0.05
Number of vocalisations	Log transformation	Repeated measures ANOVA	trials	Treatment groups	P<0.05
Latency to pass barrier	Log transformation	Repeated measures ANOVA	trials	Treatment groups	P<0.05

**(p<0.1=tendency)*

Results

General

During the randomisation process a randomisation error occurred. This resulted in unevenly divided treatment groups. Originally, the 20 pens were intended to be divided evenly across the 4 experimental groups, but instead they were divided as follows:

treatment group LDI/no larvae: 7 pens (N=70)

treatment group LDI/larvae: 3 pens (N=30)

treatment group DI/larvae: 7 pens (N=70)

treatment group DI/no larvae: 3 pens (N=30).

Needless to say, this affects the power of the entire research. Firstly, the group sizes are unequally divided. Secondly, the sample sizes of treatment group LDI/larvae and DI/no larvae are small. This makes the results less reliable compared to equal and larger sample sizes.

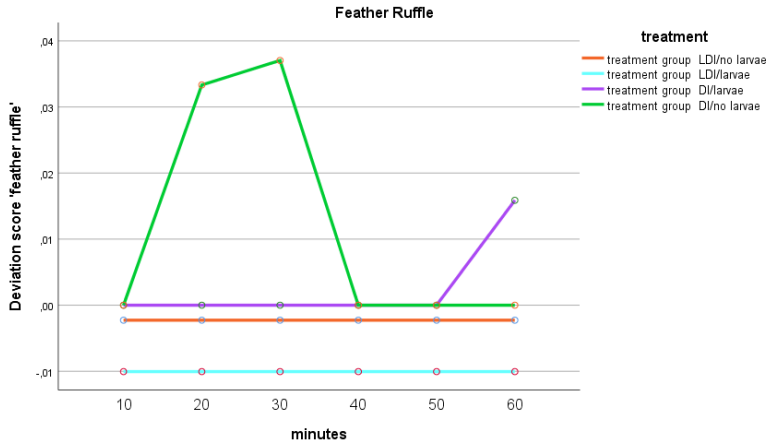
Behavioural recovery test

1a) Comparison of four treatment groups: LDI/larvae, LDI/no larvae, DI/larvae & DI/no larvae

The results of a repeated measures ANOVA show that the only significant effect of treatment can be seen for the proportion 'feather ruffle', $F(3,16) = 4.88$, $p=0.013$ (see table 2). This was the only behaviour with a significant treatment effect.

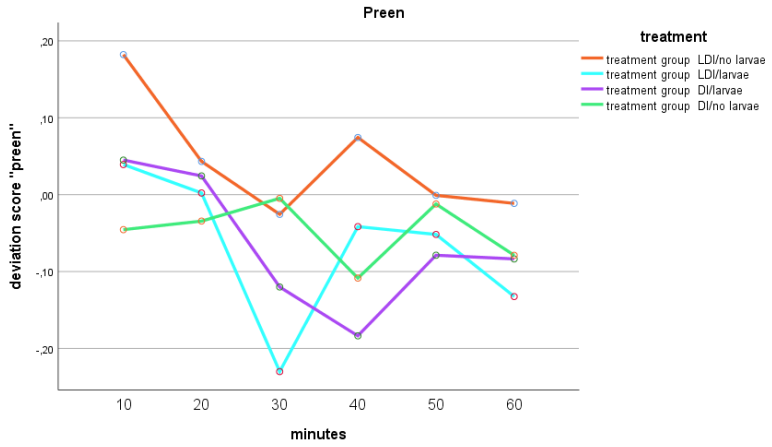
Figure 1.1 shows the four different treatment groups and their deviation score for the behaviour 'feather ruffle'. Treatment group DI/no larvae seems to show increased "feather ruffle" in the first two time-intervals compared to baseline data, after which it decreases again. The DI/no larvae group performed more "feather ruffle" compared to the other groups and did not return to baseline behaviour until time interval 4. In other words, they have a positive deviation score, which means they seem to perform more "feather ruffle" compared to baseline. Treatment group LDI/nolarvae & LDI/larvae have a negative deviation score, which could mean that the proportion chickens that showed feather ruffle on the vaccination day was lower compared to the proportion on baseline day.

Figure 1.1
Feather ruffle deviation score over six time-intervals. Each variable point illustrates the deviation score for' feather ruffle' per treatment group at a certain time-interval (minutes).



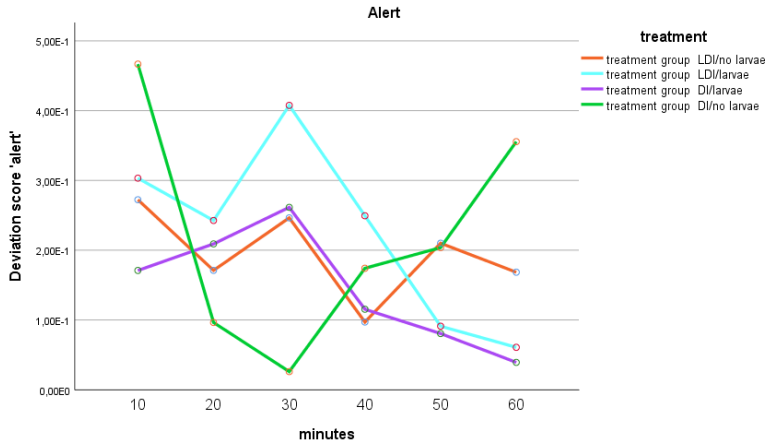
Furthermore, the results of the repeated measures ANOVA show a tendency for time effect on the behaviour variable "preen", F (5, 80) = 2.029, p=0.083 (see table 2). Fig. 1.2 shows that the proportion preening for all treatment groups on vaccination day is above baseline at first but decreases below baseline behaviour after 30 minutes.

Figure 1.2
'Preen' deviation score during six time-intervals. Every variable point represents the deviation score for 'preen' at a certain time-interval (in minutes) during the one-hour observation.



Furthermore, despite the results not being significant, in contrast to the other three treatment groups, the DI/no larvae group did almost recover to baseline for the behaviour "alert", but the deviation score increased again after 30 minutes (see fig 1.3).

Figure 1.3
Alert deviation score during 6 time-intervals. Every variable point represents the deviation score for 'alert' at a certain time-interval (in minutes).



1b) Comparison of two treatment groups separately: larvae and incubation as between-factors.

Incubation

The results show that incubation has a significant effect on "feather ruffle" F (1, 16) =13.748, p=0.002 (see table 2 & fig. 1.4). In other words, there is a significant reduction of "feather ruffle" in laying hens through exposure to light during incubation. The LDI group has a negative deviation score during all time intervals, so they performed less "feather ruffle" compared to baseline. In contrast, the DI group does not return to baseline during the hour after vaccination (see fig. 1.4), and thus shows more "feather ruffle" compared to baseline. Therefore, in other words, the LDI group did not recover to baseline, because they were already below baseline behaviour from the onset.

Figure 1.4
Deviation score of 'feather ruffle' during the time intervals for the treatment group 'incubation'. Every variable point represents the deviation score for 'feather ruffle at a certain time-interval (minutes)

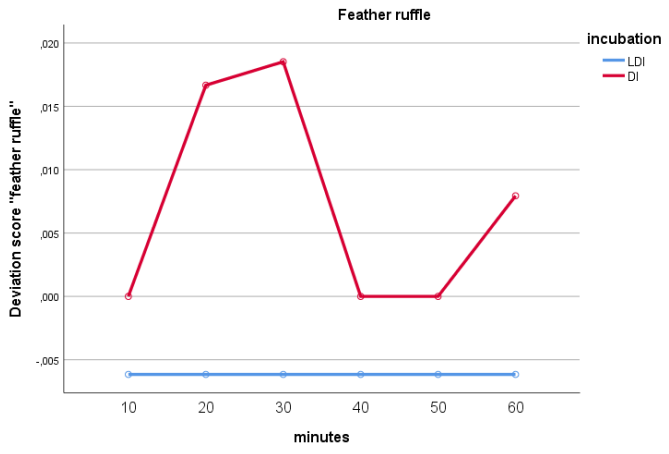
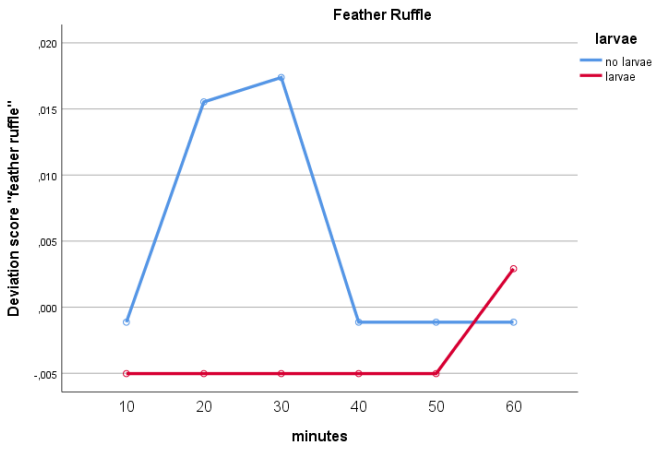


Figure 1.5
Deviation score of proportion "feather ruffle" for the treatment group 'larvae'. Every variable point represents the deviation score for 'feather ruffle at a certain time-interval (minutes)



1c) Larvae

When comparing the groups based on enrichment with larvae, a significant effect was found for the effect of larvae treatment on the behaviour "feather ruffle", F (1,16) =5.489, p=0.032 (see table 2 and fig 1.5).

Furthermore, there was a significant time x larvae effect on "alert", F (5,80) =2.571, p=0.033 (see table 2 and fig. 1.6). Both groups exceed baseline behaviour immediately after vaccination, after which the larvae group becomes less alert during the time intervals, whereas the no-larvae group becomes increasingly alert after 40 minutes compared to baseline behaviour.

Lastly, there was a significant effect for time x larvae for the deviation score 'relax', F (5,80) = 2.461, p= 0.04 (see table 2 & fig. 1.7). The larvae group almost recovers to baseline in the last 10 minutes, whereas the no-larvae group does not recover to baseline whatsoever, because it decreases even further below baseline behaviour during the last 10 minutes.

Figure 1.6
Deviation score of proportion 'alert', the effect of time x larvae treatment was found to be significant. Every variable point represents the deviation score for 'alert at a certain time-interval (in minutes).

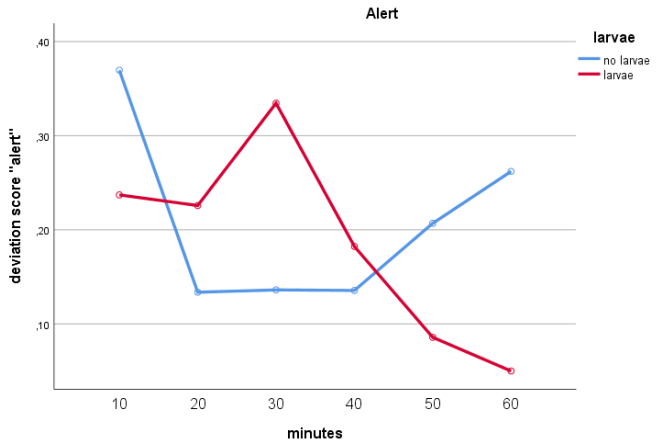
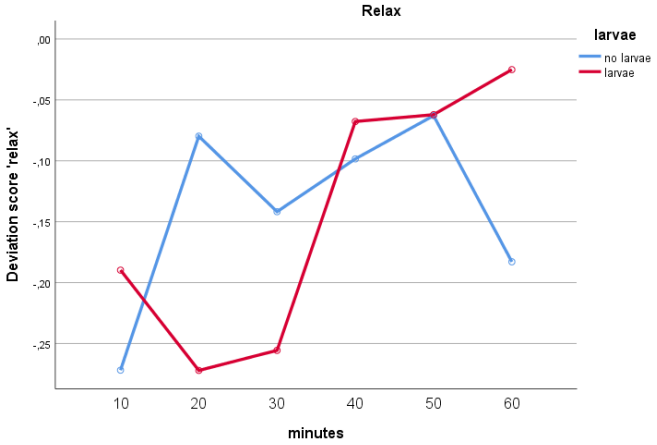


Figure 1.7. *Deviation score of proportion 'relax', the effect of time x larvae was found to be significant. Every variable point represents the deviation score for 'relax' at a certain time-interval (minutes).*

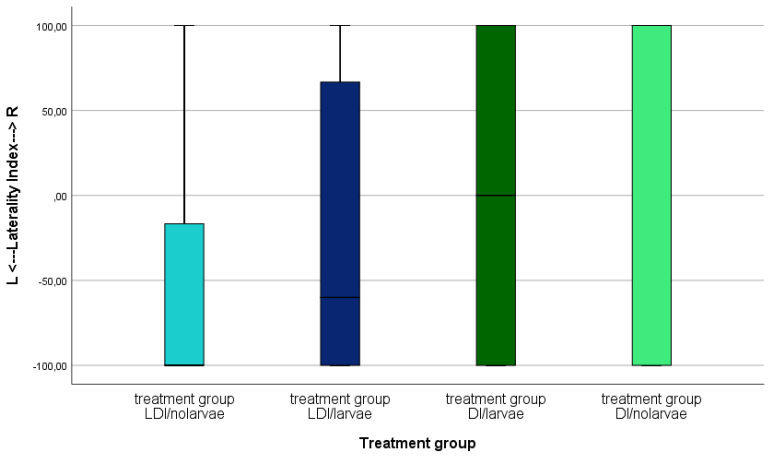


Lateralisation test

2a) Laterality Index (LI): Kruskal-Wallis

A Kruskal-Wallis H test showed that there was no statistically significant difference in laterality index score between the different treatments groups, H(3) = 3.913, p = 0.217, with a mean rank score of 26.74 for LDI/no larvae, 30.31 for LDI/larvae, 35.05 for DI/larvae and 38.67 for DI/no larvae. A boxplot shows that the LDI/larvae and LDI/nolarvae group has a stronger preference for crossing the barrier on the left, but also has more outliers (see fig. 2.1).

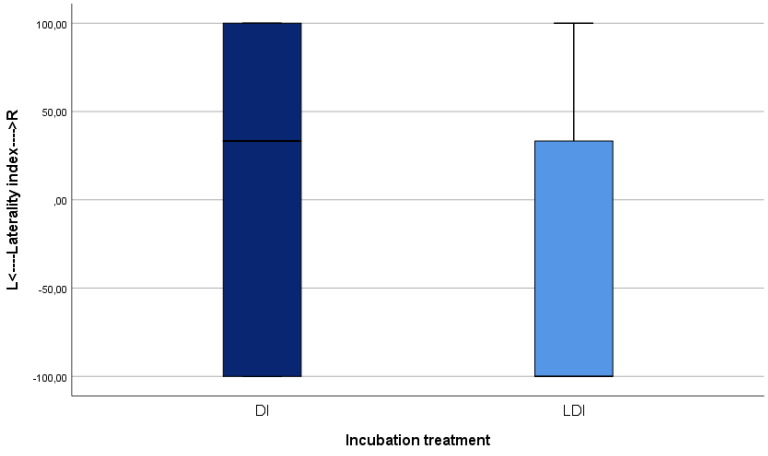
Figure 2.1
Boxplot of all treatment groups and their LI. It shows a stronger preference to the left in the +larvae group



Treatment group: incubation

A Kruskal-Wallis showed that there is a tendency of treatment effect between the LDI group and the DI group, F (1,62) = 3.386, p = 0.066 (see table 3) A boxplot shows that the LDI group has a stronger preference for crossing the barrier on the left (see fig 2.2).

Figure 2.2
Boxplot of the treatment groups apart from larvae treatment. The median shows that the LDI group has a strong preference to cross the barrier on the left



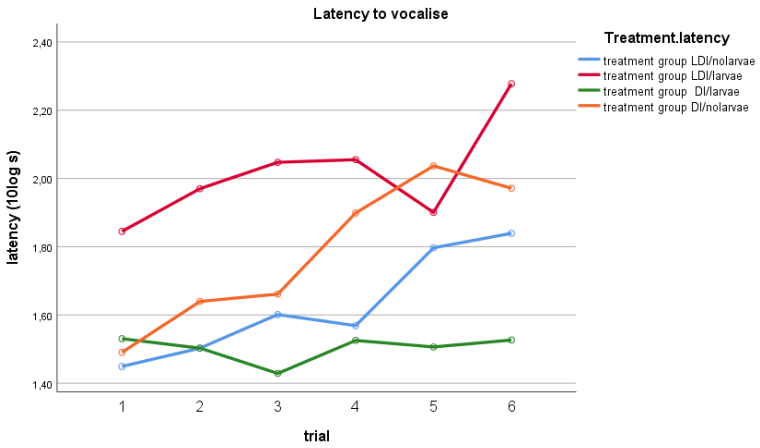
2b) Latency to vocalise

Repeated measures ANOVA

Many chicks received this maximum score (N=55), which affects the power of the test. Since it is such a high number, they cannot be considered as outliers. Therefore, we used the chicks with maximum score for our analysis. The results of a repeated measures ANOVA

show a significant effect of trials (time) on latency to vocalise, F (4.67, 354.8) = 3.028, p=0.013 (see fig. 2.3; table 3). Interestingly, the latency to vocalise increases over time (see fig. 2.3).

Figure 2.3
Latency to vocalise increases during the trials, however, the effect of treatment on latency to vocalise was not significant.

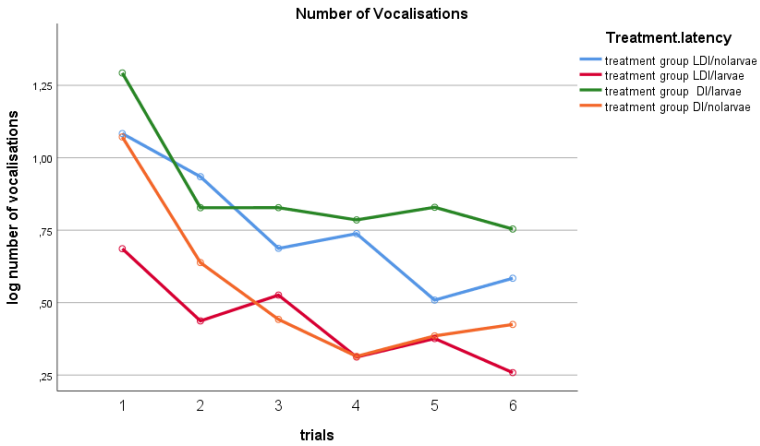


2c) Number of vocalisations

Repeated measures ANOVA

There is a tendency between treatment and number of vocalisations, F (3, 76) = 2.304, p=0.084 (see table 3). Figure 2.4 shows that there is a significant decrease in number of vocalisations during the trials, F= 12.5, p= 0.0.

Figure 2.4
Number of vocalisations during the trials. No significant effect was measured.

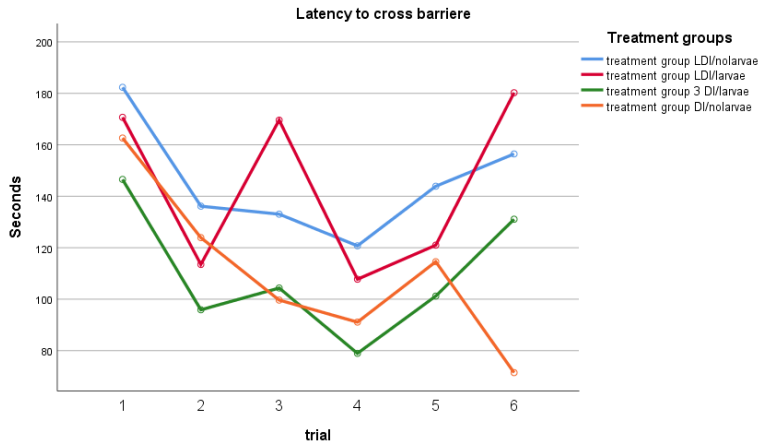


2d) Social reinstatement: latency to pass barrier

Repeated measures ANOVA

The results show no significant time x treatment effect on latency to cross the barrier. In contrast, there was a significant effect of time on latency to cross the barrier, F (4.4, 336.4) = 8.2, p=0.000 (see table 3). Latency to cross barrier can be seen in figure 2.5 for the four different treatment groups.

Figure 2.5
Latency to cross barrier for the 4 treatment groups



Mean latency to cross the barrier

As the between-subjects effects test already showed, there is no significant effect of treatment on mean latency to cross the barrier. Nevertheless, in a visual representation (see fig 2.6), the LDI group has a higher mean latency to cross the barrier, compared to the DI group.

Figure 2.6

Boxplot of mean latency to cross barrier for the four treatment groups

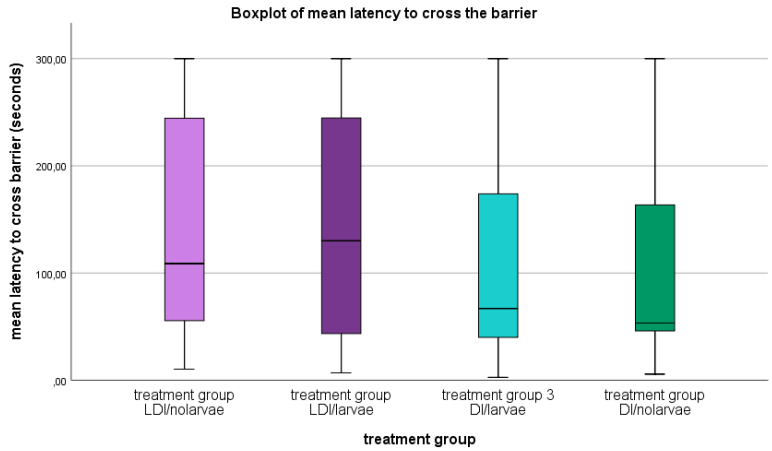


Table 2

Results of the repeated measures ANOVA on the different behaviours. Ns=not significant. Na=not available. In green are the significant results

Behaviour	Time effect			Treatment effect			Time x treatment		
	All	Incubation	Larvae	All	Incubation	Larvae	All	Incubation	Larvae
Relax	ns	na	na	ns	ns	ns	ns	Ns	F=2.461 P=0.04
Preen	F=2.029 P= 0.082	na na	na na	ns	ns ns	ns ns	ns	ns ns	ns ns
Forage	ns	na	na	ns	ns	ns	ns	Ns	ns
Feed	ns	na	na	ns	ns	ns	ns	Ns	ns
Drink	ns	na	na	ns	ns	ns	ns	Ns	ns
Dust bath	ns	na	na	ns	ns	ns	ns	Ns	ns
Alert	ns	na	na	ns	ns	ns	ns	Ns	F = 2.571 P= 0.033
Feather ruffle	ns ns	na na	na	F = 4.9 P = 0.013	F= 13.748 P = 0.002	F=5.489 P=0.032	ns ns	ns ns	ns ns
Wing flap	ns	na	na	ns	ns	ns	ns	Ns	ns
Sever feather peck	ns	na	na	ns	ns	ns	ns	Ns	ns

Table 3
Results of the different parameters of the lateralization test. Ns=not significant. Na=not applicable. In green are the significant results, in orange the results with a tendency

Lateralisation test parameter	Trial effect		Treatment		Trial x Treatment	
	F	P	F	P	F	P
Lateralisation index	Na	na	3.9	0.217	na	Na
LI: incubation	Na	na	3.4	0.066	Na	Na
Latency to vocalise	3.0	0.013	1.83	0.458	0.85	0.616
Number of vocalisations	12.5	0.000	2.30	0.084	0.85	0.608
latency to pass barrier	8.2	0.000	0.92	0.435	0.71	0.753

Discussion

Summary

All in all, most results were not significant. In our behavioural recovery test, the only significant treatment effects were found for feather ruffle. Treatment seems to decrease feather ruffle behaviour. Furthermore, there was a tendency for time effect on 'preen', which is increased after vaccination at first, and then decreases below baseline. Thirdly, the group that received larvae, relax more and are less alert compared to the no-larvae group. The results of the lateralisation test showed a significant effect of trials for latency to vocalise, number of vocalisations and lastly, latency to pass the barrier. Finally, there was a tendency for treatment effect on Laterality Index and number of vocalisations.

Behavioural recovery test

Interpretation

Conclusions about stress sensitivity are hard to draw based solely on the results of feather ruffle behaviour, but it is interesting to find out whether increased feather ruffle behaviour could be a sign of stress or at least reactive behaviour to stress. According to Eklund & Jensen's (2011) ethogram, feather ruffle is a sign of comfort. Based on that, the outcomes are not in line with the expectations.

Larvae & Incubation

Light exposure during incubation significantly reduces 'feather ruffle' behaviour. If our

hypotheses are true, and stress sensitivity is reduced through exposure to light, then feather ruffling could be interpreted as a sign of stress. Nevertheless, as stated before, it is hard to draw conclusions based on one behaviour, but it could be a possibility that feather ruffling is a coping mechanism of stress-behaviour and, therefore, light during incubation, reduces this behaviour and thus, stress sensitivity is reduced by treatment.

The group that received larvae seems to be able to relax more compared to the no-larvae group. Moreover, the larvae group becomes less alert during the time intervals compared to the no-larvae group, who become even become increasingly alert again. All in all, the larvae treatment seems to have positive effect on the welfare of young laying hens. This is in accordance with our hypotheses.

Time effect

The results could indicate that excessive preening immediately after vaccination is a sign of stress. In that sense, excessive preening could be interpreted as redirective behaviour, similar to severe feather pecking behaviour, and thus as a stress-coping mechanism. However, more research needs to be done to confirm this. According to our predictions, we expected comfort behaviour, such as preening, to increase and therefore that the hens would return or even exceed baseline behaviour, instead of the opposite. Preening is self-care behaviour, and therefore a sign of comfort (Alvino et al., 2009). Chickens that experience stress, tend to take less time for preening. The young hens seem not to be able to recover to normal behaviour after a stressor. In other words, based on these results, the effects of early-life enrichment seem not to have a positive effect on welfare of young hens, nor do they seem to affect stress sensitivity. Nonetheless, based on this result alone, it is hard to say anything about stress sensitivity or effects of early life enrichment, so therefore, we must be very careful to make statements about reduction of welfare through early life enrichment. Another interpretation could be that the behavioural recovery test was not sufficient enough to test our hypotheses properly. Thirdly, it could be an option that the power of the statistic test was too small, which consequently gives us results that are not reliable enough to base any conclusions on.

Limitations

- We looked at the treatment groups separately for “incubation” and “larvae”. Initially this was not our intention, because the treatment groups are not independent. Therefore, interpretation should be done cautiously.
- There was a bias during the vaccination day, because of the noise in the pens because of the vaccination, chickens in other pens were disturbed by this noise and were alert again. On the other hand, all the pens were affected by this bias.

Future research recommendations

For future research, in order to establish whether a certain behaviour is stress-related, it could be a possibility to measure corticosterone levels in combination with behaviour observation, after the birds receive a stressor. However, the degree of reliability of these measurements are unsure and proven that they can vary strongly among individuals (Cockrem, 2006). Nevertheless, in combination with observation, this could tell us even more about stress-coping mechanisms.

Lastly, during vaccination day the researcher had to work alone, and this made the work very hectic. Perhaps it is an idea to ask the veterinarians to work more slowly next time**.**

Lateralisation test

2a) laterality index

Interpretation

Despite the non-significant results, the visual representation shows that the LDI group tends to detour the barrier on the left, whereas the DI group tends to take a right detour. This could mean that the LDI group use their right eye more than their left, as opposed to the DI group. Especially the results of test on the incubation treatment group separately from the larvae treatment, show that the LDI group has a stronger preference for the left side. A reasonable explanation could be that the LDI group processes a fearful and novel situation with left hemisphere dominance due to lateral asymmetry, and therefore have preferential right eye use (Rogers et al., 2004). This is not what we expected beforehand, but according to another study from Rogers (2012), chicks with less lateralised brains, have difficulty in

balancing their brain functioning in terms of lateralisation. According to him, this results in a right hemisphere dominance. In other words, their left brain hemisphere has more difficulty in taking control in certain situations opposed to chicks that have more lateralised brains (Rogers & Kaplan, 2019). If this is true, that might explain why our chicks that have been incubated in the dark, have a left eye (right hemisphere) preference. Nevertheless, this topic remains very complicated, and it turns out that authors do not always agree on this point or even make contradictory statements.

If results are not significant, either treatment is not effective enough or the tests are not sufficient. Since there is much data supporting the hypothesis that a light-dark incubation has effect on lateralisation of the brain, it is probably the test in our research that is insufficient to test the hypothesis properly. Nonetheless, besides light treatment, many other factors influence lateralisation of the brain or development of fear coping mechanisms. Therefore, based on these results alone, we need to be cautious to draw any conclusions about right or left brain hemisphere dominance.

It is interesting to investigate the function and advantages of having a lateralized brain within a population. What does it mean to have a lateralised brain and to what extent does it work in your advantage of disadvantage to have a less lateralised brain? In terms of feather pecking, more research needs to be done to connect the results of this test to fearful behaviour and feather pecking. If chickens that have been hatched under a light-dark cycle are less fearful later in life, it is necessary to do follow-up research on those chicks and investigate their feather pecking behaviour and compare this to the results of lateralisation test.

According to Cockrem (2006), reactive birds tend to react more passively in fearful situations, compared to pro-active birds. They are likely to be more successful in new environments in comparison to pro-active birds, who are likely to thrive better in an environment that remains constant. If this is true, it may give us more understanding as to why so many chicks remained still and did not finish the task. This could be another interesting factor to look at in a next round.

2b) latency to vocalise

Interpretation

Increasing latency to vocalise could be the result of less fearful behaviour or because a chick froze completely. The latency to vocalise increases over time. The most likely explanation is that the chicks probably got used to the testing arena over time as well as being handled. As they got less fearful, the tendency to vocalise (a possible sign of stress behaviour) increased. Unfortunately, there is no significant difference between the treatment groups, so there does not seem to be reduction of stress sensitivity through exposure to light during incubation, nor are there any effects measured of early-life enrichment with insect larvae on the welfare of the chicks.

2c) Number of vocalisations

Interpretation

The decrease in vocalisations could be the result of the chicks getting used to being handled and placed in the testing arena, similar to the increase in latency to vocalise. On the other hand, the effect of treatment on number of vocalisations is almost significant. Interestingly, the LDI/larvae group scored lower on number of vocalisations from the onset and scored the lowest of all four groups in general. This is in accordance with our hypothesis. Therefore, it is possible that light-dark incubation and early life enrichment with insect larvae can positively affect fearful behaviour and welfare.

On the other hand, the DI/no larvae group has the steepest decline of number of vocalisations during the trials, which is not in accordance without expectations. Instead, we expected that group to have the highest number of vocalisations, and simultaneously a less steep recovery line compared to the rest of the group. However, according to Koolhaas & van Reenen (2016), stress coping mechanisms are multi-dimensional, and depend on trait characteristics such as coping style, sociality and emotionality. According to them, these individual characteristics are stable over time and can be measured in order to study their stress-coping mechanisms. Coping style can say something about the stress-vulnerability, he says, and reactive individuals tend to be more hesitant in new situations, as opposed to pro-active individuals. In accordance, an increase in vocalisations could be interpreted as a more

pro-active coping mechanism, and therefore, in line with our hypotheses. More research needs to be done in order to confirm this.

2d) Social reinstatement: latency to cross barrier

Interpretation

Interestingly, the DI/no larvae needed less time to cross the barrier than the other groups. Overall, despite the difference not being significant, the LDI group needed more time to cross a barrier compared to the DI group. This is not consistent with our hypothesis. Instead, we expected the groups that received treatment to have a shorter latency to cross the barrier compared to the groups without treatment. However, if Cockrem's (2006) statements about reactivity and proactivity are true, the DI group might be more proactive rather than reactive, which explains why they needed less time to cross the barrier and are hesitant to react to new or fearful situations. This tells us much about the way they are sensitive to stress, and, therefore, teaches us more about the effect of light treatment and early life enrichments on the welfare of young hens. Additionally, Koolhaas & van Reenen (2016) even state that reactive individuals are more flexible and would adapt easier in a new environment. The DI group would then be likely to thrive better in a stable environment, as opposed to the LDI group. Unfortunately, there is no data (yet) to confirm whether the groups show different stress-related problems in the future, such as feather pecking, as a result of the treatments.

Limitations

The lateralisation test was the first out-of-pen experience for all chicks. Many chicks froze during the first trials or during the whole experiment, and, consequently, did not finish the task or not half of the tasks (N=55). Secondly, many chicks roamed around without crossing the barrier. There was much variation in the behaviour of the chicks and how fearful they were. This makes it harder to interpret their behaviour. Many chicks were not tested, because the whole process was time consuming, so we simply ran out of time.

Nonetheless, we chose to keep the chicks that did not finish the test as data in our analyses, instead of deleting them as outliers, because they performed stress behaviour (freezing, roaming around, increased vocalising) and therefore contribute to our research question. Therefore, we set a time limit of 300 seconds, because by doing so, we keep all

chicks in the analyses. Unfortunately, the power of the test was probably too low because of the unequal sample sizes, and as a result, too small sample sizes.

Future research recommendations

- For future research, more time needs to be reserved for the testing, so more chicks can be tested. A larger population increases the power of the tests performed.
- The chicks need to get accustomed to the testing arena before official testing begins. This could decrease the number of chicks that do not finish the task. Most likely, there are better chances of the data being normally distributed, because there would not be such a high number of chicks that did not finish the task. Secondly, it will be less challenging to interpret their behaviour in the testing arena. Moreover, it could be an idea to do the trials several times during the week or several weeks. Moreover, it will give rise to more data per chick, which makes it more reliable.
- It would be interesting to compare this 'novel stimulus' response to a response from chicks that are accustomed to the testing arena. This way, it can be tested whether there is a shift from left to right eye use or vice versa in new and fearful situations compared to a less fearful situation.
- Another research variable could be added: head movement and head angle. Dawkins (2002) suggests that chickens that approach a novel object or are fearful of an object, change from large head movements to fixation and fewer head movements. Since chickens have limited eye movement, the way they move their head could tell us more about its behaviour towards a barrier, in combination with recording left or right preference. Rapid and small head movements could be the equivalent of rapid eye movements of mammals in fearful situations (Dawkins, 2002). This would be especially interesting in the case of this experiment, where the chick has not been accustomed to the testing arena prior to testing. This way, if a chick does not finish the task, we could differentiate between chicks that receives a maximum score of 300 s, based on head movement behaviour or time spend looking at an object. Instead of setting a time limit, we would then record the time a chick fixates on an object and its head movements. This could tell us even more about fear coping strategies and laterality of the brain.

- For future research, this group should receive follow-up studies in order to establish or measure whether they have different fear-coping strategies in changing environments, after treatment with light during incubation as well as early life enrichments. This way, it can be tested whether the reactivity rather than proactivity of the LDI group gives them an advantage in changing environments, as opposed to the DI group.

Conclusion

In relation to our research questions, the results are promising regarding stress sensitivity through exposure to light during incubation and the effects of early life enrichment with insect larvae. All in all, the results of the tests seem to show some effect of the treatments, especially a difference in stress-coping mechanisms. Unfortunately, the results are not significant, so more research needs to be done to confirm the effect of our treatments. Nevertheless, despite non-significant results, this study is clinically relevant. Therefore, for more reliable results, a second round of testing will be done with equal sample sizes and, therefore, more powerful statistic tests.

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