The influence of stocking density on spatial working and reference memory in laying hens (*Gallus gallus domesticus*)

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Abstract
Stocking density and housing conditions have concerned the poultry industry and animal welfare for decades and while high stocking density could have an effect on stress and stress could have an effect on learning, it is interesting to investigate the influence of stocking density on the learning ability of laying hens. Animal cognition and intelligence contain a variety of aspects that can be investigated. There have been several studies investigating the cognitive capacities of chickens, mainly focussing on associative learning, discrimination and adaptive specializations, while chickens possess a wider variety of cognition that can be analysed. Memory and spatial learning, for instance, can be assessed with a holeboard task. There have only been two published researches so far conducting a holeboard task with avians, laying hens in particular. Therefore, the aim of this study was to examine the influence of stocking density on spatial working and reference memory in laying hens (*Gallus gallus domesticus)* to test the hypothesis that chickens housed with a lower stocking density will have better spatial working and reference memory than chickens housed with a higher stocking density. This study also aimed to find a more adequate method of holeboard training and a more adequate holeboard cup model for laying hens than the previous studies and it aimed to correct for spatial pattern learning. 39 laying hens were used in this experiment, separated into two groups of 5, one group of 14 and one group of 15 hens, randomly selected. Out of each group 5 test subjects were randomly selected to make a sample size of 19 laying hens: 10 representing high density (1,75 x10^{-8}/cm^2 and 1,88 x10^{-8}/cm^2) and 9 representing low stocking density (6,25 x10^{-9}/cm^2). The results showed no major density effect for working or reference memory. This study demonstrated a more adequate holeboard task with a better cup model resulting in a learning curve for reference memory for both high and low density and high working memory scores.

Keywords: Laying hen, reference memory, learning memory, hole board, cognition, spatial learning

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1. Introduction

Stocking density and housing conditions have concerned the poultry industry and animal welfare for decades. Stocking density has an influence on chicken behaviour and physiology like serum corticosterone concentration, stress, feather pecking and aggression and it has been found that high stocking density increases feather pecking and that the corticosterone concentration of hens housed in a more crowded cage is consistently higher than that of hens in a less crowded cage (MASHALY et al., 1984; Nicol et al., 1999). Feather pecking and raised corticosterone concentrations are indicators for stress and stress can affect the learning capacity of chickens (Lindqvist, 2007).

Relatively little is known about the cognitive capacities of chickens even though there are 19 billion chickens worldwide according to the UN Food and Agricultural Organisation (2011). Cognition and intelligence contain a variety of aspects that can be investigated. A recent review by Marino (2017) describes scientific data on cognition, emotions, personality, and sociality in chickens. The author brings forward that chicken cognition is more complex than we might think. A variety of aspects of cognition is being discussed, describing visual cognition and spatial orientation, numerical abilities, time perception and anticipation on future events, reasoning and logical interference, self-awareness and social cognition and complexity. According to the review, chickens possess capacities including all aspects addressed before, but most articles have been focussing on discrimination, associative learning and adaptive specializations. Croney et al. (2007), for example, examined the learning ability of chickens with social dominance as the discriminating factor. A visual discrimination learning task had been used to investigate the learning ability of New Hampshire domestic roosters (*Gallus gallus*). In another experiment with social dominance as a factor, chickens were trained to finish a maze in order to investigate their learning capacity (Candland and Conklyn, 1968). Both researches collected data on cognition and their relation to dominance and they differ in what aspect of cognition was examined, but the main focus was still a form of discrimination or associative learning, since Croney et al. investigated visual discrimination and the experiment performed by Candland and Conklyn investigated the capacity to learn a maze.

An aspect of cognition that has not frequently been investigated is memory combined with spatial learning. These cognition aspects give an insight in the spatial orientation and time perception of an animal (Marino, 2017). Spatial working and reference memory are considered forms of short-term and long-term memory, respectively, and these aspects have been analysed in non-avian species with a holeboard experiment (van der Staay et al., 2012). The first published article that utilised a holeboard task to investigate spatial working and reference memory in avians, specifically laying hens, has been performed by Nordquist et al. (2011). Another study, performed by Tahamtani et al. (2015), used a holeboard task to determine whether differences in early life environmental complexity have an impact on spatial cognition and memory and found that laying hens that had been reared in a barren environment had long-term impairment of short-term memory. These two studies have been the only researches so far utilising a holeboard test to obtain more knowledge about the cognitive capacities of chickens. More research needs to be done to investigate other factors that could have an influence on the memory capacities of chickens and it is important to enlarge the knowledge about chicken cognition in general to obtain more data on chicken cognition for comparison and discussion. Besides, the study of Nordquist et al. (2011) states that it is important to obtain more data on memory in chickens of different ages to investigate if the birds inherently show poorer reference memory than other species. Their study also suggests other testing setups to improve experiments on memory in chickens, referring to the long duration of the learning process in the birds. Therefore, in the present research, the chickens will be older, the model of the holeboard cups will be improved and the training sessions will be slightly different from the training phases described by Nordquist et al.. The present research also aims to correct for spatial pattern learning in order to avoid egocentric spatial learning (relative to itself) and focus on allocentric spatial learning (relative to the environment) (van der Staay et al., 2012).

The aim of this study was to examine the influence of stocking density on spatial working and reference memory in laying hens (*Gallus gallus domesticus)* to test the hypothesis that chickens housed with a lower stocking density will have better spatial working and reference memory than chickens housed with a higher stocking density.

2. Materials and methods

2.1. Test subjects and housing

The animals that were used in this experiment were 39 laying hens *(Gallus gallus domesticus)*, Novo Brown Light. The eggs were hatched at Verbeek Hatchery Holland and the chickens were reared at Landbouwonderneming Bouwland. At about 17 weeks of age, the chickens were transported to their housing at the Tolakker in Utrecht, where the experiment was conducted. The chickens were about 30 weeks of age at the start of the experiment. They were separated into two groups of 5, one group of 14 and one group of 15 hens, randomly selected. The two groups consisting of 5 hens represent low stocking density (6,25 $x10^{-9}/cm^2$) and the groups with 14 and 15 hens represent high stocking density (1,75 $x10^{-8}/cm^2$ and 1,88 $x10^{-8}/cm^2$ respectively).The groups were housed in four identical, adjacent pens of 200cmx400cm, separated by chicken wire covered with canvas (150cm high) which the birds could not see through. Each pen contained 3 to 4 nest boxes. The floor of the pens was covered with chopped straw (Appendix 1). All four groups received the same feed and water (De Heus Voeders BV, Legkorrel Scharrel, the Netherlands). Feed and water were both provided *ad libitum*. During the day the birds were exposed to natural daylight, with the addition of one lamp above each pen that was switched on in the morning before sunset and extra lights in the barn that were switched on when the experimenters were present, usually from 08:30 h to 16:00 h. Light intensity in the barn was measured at the start (42,4 lux at 09:30 h and 10,05 lux at 17:00 h) and end of the experiment (5,7 lux at 17:00 h, data for 9:30 measurement is unavailable). The pens were naturally ventilated. At the start of the experiment, mean temperature in the barn was 8°C, mean humidity was 13% , sunrise was at 07:35 h and sunset was at 17:10 h. At the end of the experiment, mean temperature was 5°C, mean humidity was 7% , sunrise was at 08:39 h and sunset was at 16:33 h.

2.2. Holeboard test

2.2.1 Holeboard apparatus

The holeboard was set up in a chamber with concrete walls and floor (285x315 cm). Nine cups were placed in a 3x3 matrix with the centre cup placed in the centre of the room and the cups standing in equal distances from each other (Fig 1). Several changes were made to the original holeboard design used in a previous study by Nordquist et al. (2011). The nine cups that were used in this experiment were a renewed model of the cups used in that study. In short, the renewed cups were shaped like a cube with a triangle shaped top with a swing lid to allow chickens to peck at the lid, consume the bait and leave the cup with the lid swung back into position (Fig 2). Also, the holeboard used by Nordquist et al. included approach circles, which were omitted in this study because of the renewed cup model and a lack of space.

Each chicken was assigned to its own configuration of 3 baited cups. The bait was a piece of grape (approximately 2cm^2). In order to correct for spatial pattern learning, four starting points were used. This was accomplished by placing a chicken in one of four cylindrical mesh cages (50x40 cm), placed in each corner of the holeboard (Appendix 2). The mesh cage could be lifted by pulling the end of the wire in the monitoring room situated next to the holeboard room, allowing the chicken to explore the holeboard arena. Two cameras were placed above the holeboard to record the test subject and its behaviour during the sessions.

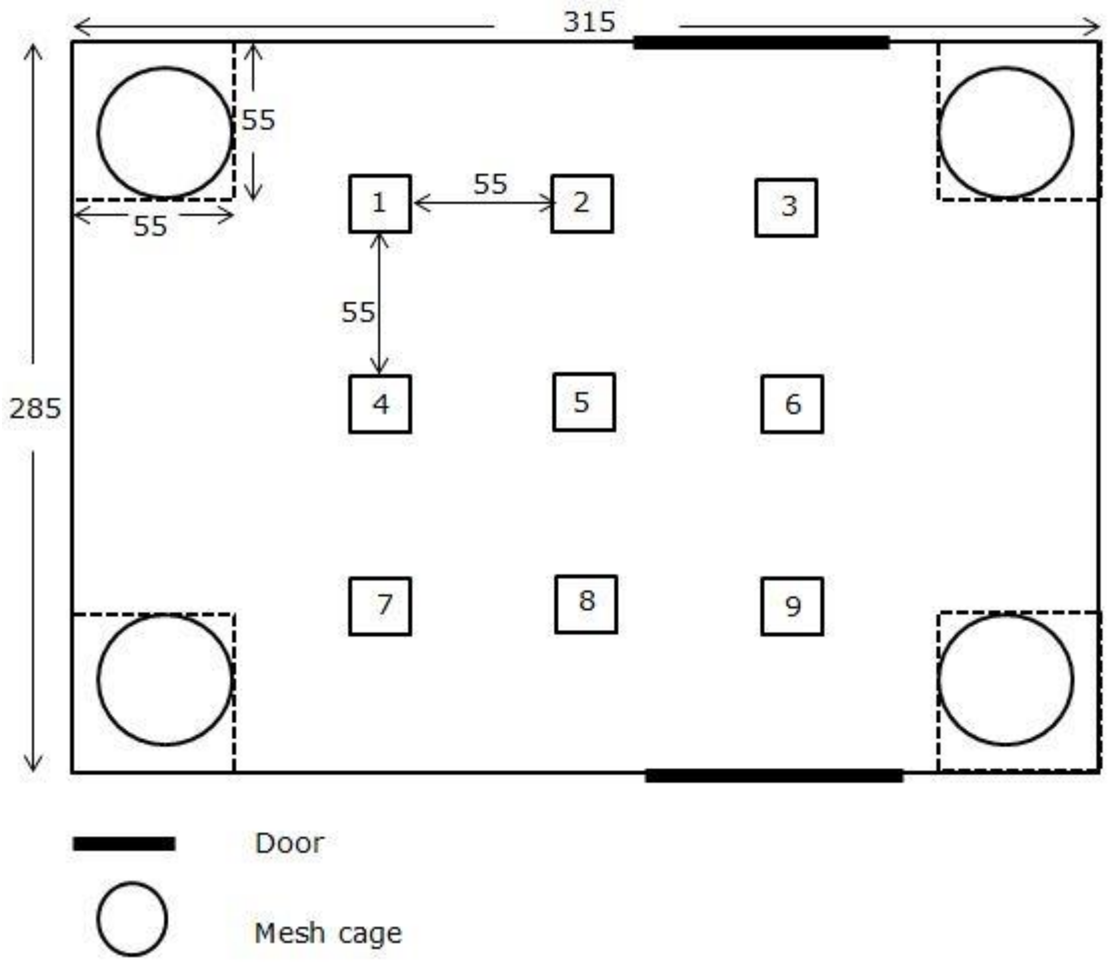

Fig. 1. Holeboard apparatus. Each measurements are in centimetres. Each chicken was assigned to its own configuration of 3 baited cups. The bait was a piece of grape (2cm^2). Four cylindrical mesh cages (50x40 cm) were used as starting points. Each cage was placed in a corner of the holeboard room.

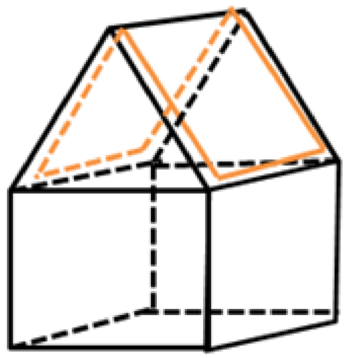

Fig. 2. Schematic drawing of holeboard cup. Renewed holeboard cup model with a swing lid (orange lines). Each cup contains a false bottom with a piece of grape underneath. Only the baited cups have an additional piece of grape on top of the false bottom.

2.2.2. Habituation period

4 weeks prior to the holeboard sessions the hens received a leg ring and were randomly divided over the four pens until the desired stocking densities were reached. 5 chickens were randomly selected out of each pen to make a total of 20 individual test subjects: 10 test subjects representing low stocking density and 10 test subjects representing high stocking density.

In the 4 weeks prior to the start of training, the chickens were handled by the researchers in order to get used to the presence of and handling by the researchers. The handling took place 3 times per week and consisted of weighing the hens in their home pens. In addition, the chickens had to be familiarized with used food reward and the holeboard cup. To accomplish habituation to these objects the chickens were offered grapes and presented a model of the holeboard cup in their home pens 3 times per week for the first 2 weeks of these 4 preparation weeks. In the last 2 weeks the test subjects were placed in the holeboard room and put back in their home pen 5 times per week, once a day, for max. 5 minutes. During this period only one of the 4 mesh cages was used as a starting point and contained a single cup with a piece of grape placed close to the starting cage (Appendix 3).

2.2.3. Holeboard experiment

After 4 weeks of habituation, training in the holeboard started. The holeboard experiment consisted of 3 phases: a cued phase, an uncued phase and a reversal phase. Each test week included 5 session days with 2 sessions per day (one in the morning and one in the afternoon) for each individual chicken. The cued phase consisted of sessions with a consistent configuration (randomly assigned for each test subject) of three baited cups cued with an LED. The second phase had the same configuration of baited cups, but without the LED cues. The third phase was a reversal phase with a different configuration of baited cups (randomly assigned for each test subject). A new phase started after all chickens had reached criterion performance, which was a reference memory score of 0.6 or higher at least three times in four consecutive sessions. For practical reasons, transitions to a new phase were done simultaneously for all chickens (Nordquist et al., 2011).

The chickens were not food-restricted prior to a session. A session always begun at one of the four starting points, determined at random, and a session was stopped after all three baited holes had been visited or after five minutes from the start of the session, whichever occurred first.

All chickens were weighed and received a general health check once a week during the experiment.

2.2.4. Deviations

After the first five sessions of the cued phase, design of the cups was adjusted as it was noticed that the test subjects were able to visually check for the presence of a food reward through the gap between the lid and the cup. Therefore, the wooden false bottom was replaced with a transparent plastic bottom so that the chickens had a visual (and olfactory) cue for a piece of grape in every cup, but only in the baited cups (with a another piece of grape on top) they had access to the grape. This resulted in a total of four phases (instead of three): phase 1.1 (cued and wooden false bottom), phase 1.2 (cued and plastic false bottom), phase 2 (uncued and plastic false bottom) and phase 3 (reversal, uncued and plastic false bottom).

Furthermore, after nine days chicken number 10 (not a test subject) was moved from pen 1 and placed in a separate cage within pen 3, because of a physical trauma. This resulted in a total number of 14 chickens in pen 1 and 6 chickens in pen 3. The chickens in pen 3 could interact with the isolated chicken, but it was housed within a mesh cage (Appendix 4). Twenty days later the isolated chicken was moved to another owner, but chicken number 19 was then placed into the mesh cage in pen 3, also because of a physical trauma.

Finally, Chicken number 12 was excluded from the test after ten days, because it did not portray any foraging behaviour in the holeboard apparatus. The data of number 12 was not used for the statistical analysis resulting in a total sample size of 19 with 10 test subjects for high density and 9 test subjects for low density.

2.3. Ethical approval

Experiments were reviewed by an Animal Experimental Committee and determined to fall outside of the European Regulations for required approval by the Dutch National Authority due to absence of suffering for the animals.

2.4 Holeboard measures

The following measures were scored per session (Nordquist et al., 2011):

Reference memory (RM): RM was described as the number of visits to the baited set of holes divided by the total of visits to all holes. RM gives information about long term memory.

Working memory (WM): WM was described as the number of rewards found (with a maximum of 3 since there is a set of 3 baited holes) divided by the total number of visits to the baited set of holes. WM gives information about short term memory and defines the ability of the test subject to avoid re-visits to a baited cup.

General working memory (GWM): GWM was described as the number of unique holes visited (with a maximum of 9 since there were 9 different holes) divided by the total number of visits to all holes. GWM gives information about short term memory and the ability of the test subject to avoid re-visits to all cups.

Latency first peck (LFP): LFP was defined as the time measured in seconds until the test subject's first peck at any of the nine holes (with a maximum of 300 seconds).

Latency first peck at baited cup (LPBC): LPBC was defined as the time measured in seconds until the test subject's first peck at any of the three baited holes (with a maximum of 300 seconds).

Trial duration (TD): TD was defined as the total duration of a session in seconds (with a maximum of 300 seconds).

Total number of visits (TV): TV was defined as a total number of visits to all holes within a session.

Number of unique holes visited (UHV): UHV was defined as the number of unique hole visits (with a maximum of 9).

Each peck at a cup was scored as a cup visit, irrespective of the exact location of the peck (i.e. as long as the beak made contact with any surface of the cup, it was scored as a visit). The chicken had to make at least two steps away from the cup for a following peck at the same cup to be counted as a re-visit.

2.5. Statistical analysis

All statistical analyses were performed using R for Windows (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA) .

For all variables, means of two successive sessions (session blocks) were calculated across all four phases. A mixed model analysis was used to analyse the effect of density on the variables with session block and density as fixed effects and chicken nested in pen as random effect. An autoregressive (1) correlation structure was used to account for repeated measures. All variables were analysed per phase with phase 1.1 consisting of 7 blocks (session 1-14, block 1-7), phase 1.2 consisting of 6 blocks (session 15-26, block 8-13), phase 2 consisting of 11 blocks (session 27-48, block 14-24) and phase 3 consisting of 5 blocks (session 49-58, block 25-29). Mixed models were also used to analyse the impact of a transition to a new phase with block and density as fixed effects and chicken nested in pen as random effect. The means of the last block of one phase and the first block of the following phase (together it represents the transition) were used in this analysis.

Distribution of residuals was assessed with a scatterplot, a qqnorm and a boxplot. Latencies and trial durations were transformed with a reciprocal transformation (1/x) to improve distribution of residuals.

For all results, a value of $p<0,05$ was considered statistically significant.

3. Results

3.1. Reference memory

Reference memory (RM; see Fig. 3) increased significantly in phase 1.1 for both high and low density (Block: $F(6,108)=16.2258$, $P<.0001$). Besides, there was an increase in phase 1.2 (Block: $F(5,90)=9.6405$, $P<.0001$) and phase 2 also showed an improved RM (Block: $F(10,108)=2.9111$, $P=0.0021$). The reversal (Phase 3) did not show a significant improvement (Block: $F(4,72)=1.2476$, $P=0.2986$), but the graph shows an increasing line for high density and a decreasing line for low density. There was no density effect found on RM for all four phases.

As for the transitions, all three transitions showed an increase in RM (Transition 1.1-1.2: Block: $F(1,18)=12.9234$, $P=0.0021$; Transition 1.2-2: Block: $F(1,18)=7.0579$ $P=0.0161$; transition 2-3: Block: $F(1,18)=17.8543$, $P=0.0005$). Only the transition from uncued to cued (Transition 1.2-2) showed an effect of density (Dens: $F(1,17)=7.6182$, $P=0.0134$). The average RM for low density was 0.13 lower than high density.

3.2. Working memory

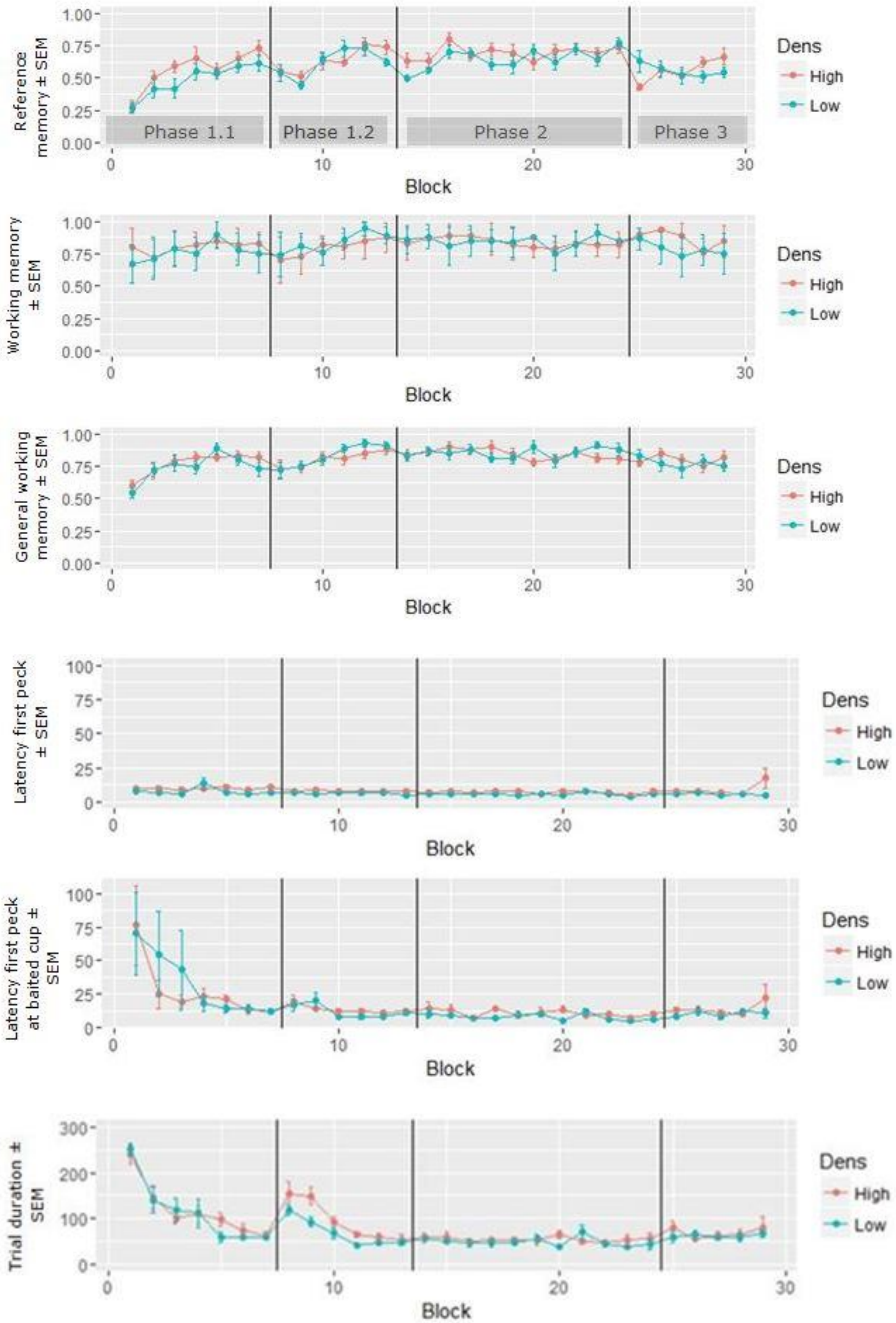

Fig. 3. Performance of high and low density groups. Session blocks scores for reference memory, working memory, general working memory, latency first peck, latency first peck at baited cup, trial duration, total number of visits, number of unique holes visited. There were four phases: Phase 1.1 (cued, wooden false bottom), Phase 1.2 (cued, plastic transparent false bottom), Phase 2 (uncued, plastic transparent false bottom) and Phase 3 (reversal, uncued, plastic transparent false bottom). Vertical black lines indicate the transitions.

Only phase 1.2 showed a significant increase in working memory (WM; see Fig. 3) (Block: F(5,90)=4.1954, P=0.0018). None of the phases showed an effect of density and the graph shows an improved performance of WM starting with phase 1.1, leading to a plateau-level at phase 2. The lowest block average of WM was between 0.65 and 0.7 and occurred in block 1. The transitions showed no effect of density or block.

3.3. General working memory

General working memory (GWM; see Fig. 3) improved the first two phases (Phase 1.1: Block: F(6,108)=6.6992, P<.0001; Phase 1.2: Block: F(5,90)=6.3631, P<.0001). The reversal had no significant impact GWM. There was no effect found for density. There was no significant change in GWM for the transitions and there was no effect of density.

3.4. Latency first peck

For latency to first peck (LFP; see Fig. 3) there was a block effect found for phase 2 (Block: F(10,180)=1.95241 P=0.0410). The graph does not show an increase or decrease in latency, but there were some outliers with a significantly lower LFP. The transitions did not show an effect of block. No density effect was found on latency first peck for any phase or transition.

3.5. Latency first peck at baited cup

The chickens became faster in phase 1.1, 1.2 and 2 and showed a decrease in latency first peck at baited cup (LPBC; see Fig. 3) (Phase 1.1: Block: F(6,108)=2.32944, P=0.0373; Phase 1.2: Block: F(5,90)=2.64556, P=0.0281; Phase 2: Block: F(10,180)=1.91794, P=0.0453). The reversal shows an increase in LPBC in the graph, but this was not a significant effect (Block: F(4,72)=0.58061, P=0.6776). The transitions showed no block or density effect.

3.6. Trial duration

Phase 1.1 and 1.2 showed a decrease in trial duration (TD; Fig. 3) (Phase 1.1: Block: F(6,108)=10.5330, P<.0001; Phase 1.2: Block: F(5,90)=17.6888, P<.0001), but no density effect. There was no block or density effect found for phase 2 and 3. The first block of phase 3 shows an increased trial duration compared to the average of phase 2, but only transition 1.1-1.2 showed an effect of block (Block: F(1,18)=47.29669, P<.0001) with a longer TD at the beginning of phase 1.2 than the TD at end of phase 1.1.. No density effect was found for the transitions.

3.7. Total number of visits

Both phase 1.1 and 1.2 show a decreasing line for the total number of visits (TV; see Fig. 3) in the graph and this was a significant block effect (Phase 1.1: Block: F(6,108)=3.4287, P=0.0039; Phase 1.2: Block: F(5,90)=11.36867, P<.0001). No density effect was found for any of the four phases. There was a significant difference in TV for transition 1.1-1.2 (Block: F(1,18)=5.70032, P=0.0281) and for transition 2-3 (Block: F(1,18)=6.1990, P=0.0228). No difference between high and low density was found for TV.

3.8. Number of unique holes visited

Both phases 1.1 and 1.2 have a decreasing number of unique holes visited (UHV; see Fig. 3), but only phase 1.2 had a significant effect (Block: F(5,90)=11.5959, P<.0001). Transition 1.1-1.2 and transition 2-3 also showed a block effect (Transition 1.1-1.2: Block: F(1,18)=12.8175, P=0.0021; Transition 2-3: Block: F(1,18)=12.9670, P=0.0020) with an increased UHV at the beginning of the new phase. There was no density effect found for any of the four phases or any of the transitions.

4. Discussion

In this study, the effect of stocking density on spatial working and reference memory was analysed in a group of laying hens. The hypothesis was to find a higher spatial working and reference memory for low stocking density, but there was no density effect found for both working and reference memory within the four phases. This means that cognitive performance for both densities increased at the same rate and reached about the same plateau-level at the same time. On the other hand, there was a density effect for transition 1.2-2, resulting in an average reference memory for low density that was 0.13 lower than that for high density. There are a few possible explanations for the dominate absence of a density effect for this study. First of all, the experimental design and methods regarding housing and density could have been not determinative enough to actually show density effects. Most researches that have been done with high and low densities concerning differences in stress levels and stress indicators like feather pecking, were conducted with considerably higher stocking densities for both low and high density compared to the present study (Hughes et al., 1997; MASHALY et al., 1984; Nicol et al., 1999). Besides, although the hens were not able to directly see each other because of the canvas, they could still hear each other. This could have reduced the actual effect of being separated into groups. It is recommended for a future study to take more measures for separating groups with different densities and to create densities that are comparable to previous studies and perhaps the modern poultry industry for more relevant and reliable results.

A second possible explanation for the absence of a density effect in the current study could be that the presence of stress for both densities was uncertain. There were no additional measurements for stress indicators such as corticosterone concentration, feather pecking or aggression. For a following study that concerns stocking density and stress, it is recommended to also measure stress indicators.

Besides the test methods that could be the cause of absent density effects, as discussed above, literature has no conclusive arguments for the correlation between stress and preference for a certain density or group size nor for the effect of stress on cognition. This indicates that it is difficult to find density effects that are relevant for the knowledge on chicken cognition. The study of Nicol at al. (1999), for instance, found that plumage condition worsened with increasing stocking density, while the aggressive form of feather pecking was found for the low stocking densities. Low aggression for high stocking density was also found by Hughes et al. (1997). As for corticosterone levels, it has been found that high density results in consistently higher serum corticosterone concentrations in laying hens (MASHALY et al., 1984). A study that analysed group size preferences in chickens showed that small groups are preferred, but sufficient space is an important factor. They also suggest that preference for a certain group size depends on a hen's dominance status (Lindberg and Nicol, 1996). Furthermore, a study about stress, brain plasticity and housing conditions found that certain brain structures that play a role in stress experience were only mildly effected by the different housing, suggesting that adult brain plasticity is low (Patzke et al., 2009). This could indicate that the chickens used in the current study were too old to be affected by the stocking density differences and possible presence of stress. In short, possible explanations for the absence of a density effect in the current study regarding the four phases are the experimental design and methods and the fact that there could be no density effect in general, according to non-conclusive literature findings.

Overall, it was found that the chickens have the ability to learn the holeboard task, since reference memory (RM) showed an increasing line during the acquisition for both high and low density. This result is in line with the outcome of the research of Nordquist et al. (2011). They also found an increasing line for RM although this line increased slower than the RM line in the present study. A possible explanation is the fact that their cued phase was preceded by an uncued phase which probably lead to a slower acquisition of the task by the chickens. Another difference is the maximum average RM. In the present study this variable reached levels between 0.7 and 0.8 while the study of Nordquist et al. showed maxima between 0.6 and 0.7. The slower increase and lower maxima of that study could indicate that that the method of the present study and the renewed model of the cups are an improvement and result in a more adequate holeboard task for laying hens that gives more information about their actual cognitive capacities.

In the present study, working memory (WM) showed a significant increase for phase 1.2 and the graph showed an increase over time starting at phase 1.1 (not significant) that reached plateau-level at phase 2. The lowest block average of WM was between 0.65 and 0.7. Something similar was found for the holeboard experiment of Nordquist et al. (2011) where there was a slowly increasing line for WM that reached plateau-level at the overtraining phase (similar to phase 2 of the present study). It can be noted that the WM minimum of the present study is higher than the minimum of the study of Nordquist et al.. This indicates that the chickens were less motivated to re-visit a baited cup in the present study. This could be explained by the renewed model that needs a high motivation to be utilised by a chicken. These findings also suggest that the present study is an improvement of previously conducted holeboard task with laying hens.

The results show that general working memory (GWM) increased in phase 1.1 and 1.2 similar to WM, but these increases were both significant block effects while WM only showed a significant block effect for phase 1.2. This was probably the result of a lower minimum in the first sessions of phase 1.1 compared to the minimum of WM. This implies that re-visits to the baited set of holes happens less frequently than re-visiting any of the nine cups (baited and none-baited). Nordquist et al. (2011) found a strong correlation between WM and GWM and stated that, together with the high WM and GWM scores, this would support the nature of a chicken’s foraging behaviour as a win-shift/loose-shift strategy. The findings for WG and GWM of the study of Tahamtani et al. (2015) were also in line with that study and the present study. WM will be high as well in other species that use the same foraging strategy (van der Staay et al., 2012). In a holeboard task with pigs, for instance, there were WM scores found between 0.6 and 1.0, with the lowest scores at the beginning of the reversal phase (Roelofs, 2017). In rats, WM resulted in scores between 0.7 and 1.0 (van der Staay, 1999). Both species show a win-shift/loose-shift strategy that can be compared to the WM scores of the laying hens in the current study and the previous ones (Nordquist et al., 2011; Tahamtani et al., 2015).

As for the transitions, all three phase transitions resulted in a significant difference for RM over time. Combined with the noticeable decline of RM at the start of a new phase, it can be said that the difference in phases does have an effect on cognitive performance and that the chickens found difficulties transitioning. When it was noticed that some of the chickens took advantage of the gap between the lid and the cup, the wooden false bottom was replaced with a plastic transparent false bottom. This change was necessary for more reliable results and resulted in transition 1.1-1.2. Since this transition had a significant block effect, it could be that the change in false bottoms did solve the problem. As for transition 1.2-2, there was a change from cued baited cups to uncued baited cups. This transition also showed a significant block effect and suggests that the chickens actually used the LEDs as a visual discrimination for which holes were baited and which were not. The transition from phase 2 to phase 3, the reversal, there was also a block effect. This shows that the chickens had learned the position of the cups of their original configuration, because there was a declined spatial cognition performance at the start of the reversal phase. Even though the chickens were able to find the baited cups from each of the four starting points, this is not a solid conclusion for allocentric spatial learning. The chickens could still have learned a spatial pattern associated with the position of the starting cage.

Referring back to the significant increase in RM for the acquisition phases and a block effect for all three transitions, it can be stated that these laying hens adapt quickly to changed settings in the holeboard resulting in acquisition of the task in every phase.

Additional variables were analysed to assess the cognitive ability of laying hens in a holeboard task. Measured latencies were analysed for additional information about motivation and effectiveness. The results show a low latency to a first peck (LFP) for all phases with no noticeable block effect, which could indicate that the motivation of the chickens to peck at the cups was high and that the habituation period was effective. Phase 2 did result in a significant block effect, but when it is being compared to the actual graph, there is no increase or decrease in the lines. There were some variable results across time that may have resulted in this significant effect, i.e. alterations in test results on the Monday following an antagonistic interaction between the chickens over a weekend. The average LFP was significantly lower on this day, compared to the other days.

While a relatively low and constant LFP suggests high motivation and a successful habituation, a significant block effect for latency to a first peck at a baited cup (LPBC) for phase 1.1, 1.2 and 2 suggests improvement of effective seeking behaviour and cognitive performance. The opposite was to be expected and reflected by the reversal phase where LPBC was increased. The chickens were assigned a different configuration of baited cups for the reversal, so the chickens needed more time to find a baited cup which lead to the increased LPBC. In a holeboard study with pigs, it was also found that latency to a first baited hole decreased during acquisition and reversal, with an increase at the start of the reversal phase (Roelofs, 2017).

The increased LPBC for the reversal in the present study had no effect on trial duration (TD), since TD had no significant block effect for phase 3 or transition 2-3, but the graph shows an increased TD at the beginning of phase 3. Nordquist et al. (2011) and Tahamtani et al. (2015) found similar results with laying hens. Even though TD showed no significant block effect for transition 2-3, the reversal did have an effect on seeking behaviour in the beginning of phase 3 since there was a significant block effect found for transition

2-3 for total number of visits (TV) and number of unique holes visited (UHV). This suggests that the chickens visited more cups compared to the end of phase 2 and that there was a motivation to visit a more diverse range of unique cups.

As stated before, RM shows a significant block effect for transition 1.1-1.2 and suggests that the replacement of the false bottom had an effect on the chickens' seeking behaviour and cognitive performance. The additional variables also give information about this transition when phase 1.1 and 1.2 and transition 1.1-1.2 are being compared. As for TD, phase 1.1 did show a block effect with a decreasing TD. This leads to the assumption that the chickens had trouble finding all the baited holes at the first phase, but managed to learn their configuration and reach a significantly low TD time within the same phase. Phase 1.2 and transition 1.1-1.2 also showed a block effect. If the change of the false bottom had no effect, phase 1.2 should have shown no increase or decrease compared to phase 1.1 and no block effect. Phase 1.2 started with a significantly increased TD compared to the end of phase 1.1, but decreased over time. This suggests that the change to a plastic false bottom had an impact on the chickens cognitive performance and seeking behaviour, but acquisition of the task was still possible. In addition, analysing the results of TV for phase 1.1 and 1.2 and transition 1.1-1.2, it can be found that there was a significant block effect for this variable and these sections as well, which supports the assumption that the plastic false bottom had an impact on cognitive performance and seeking behaviour, because there was decrease over time with a significantly higher TV at the beginning of phase 1.2 compared to the end of phase 1.1. Besides, since visiting more holes takes more time, TV could be an explanation for the block effects for the variable TD. On top of that, the significant block effect for UHV for transition 1.1-1.2 and a declining line in phase 1.1 and 1.2 (although only significant for phase 1.2) could be an argument for an effected seeking behaviour and cognitive performance as well. The chickens showed a low UHV at the end of phase 1.1, but this was increased significantly at the start of phase 1.2. This indicates that the chickens were motivated to check more unique cups after the transition on order to obtain all the baites.

5. Conclusion

The current study found no major differences for any of the variables between the groups housed in different densities. So, according to this study, the hypothesis that chickens housed with a lower stocking density will have better spatial working and reference memory than chickens housed with a higher stocking density can be rejected. Although, the materials and methods used in this study are more adequate for laying hens and showed better cognitive performance scores then previous studies, it is recommended to conduct more holeboard studies with laying hens and to improve testing setups for more reliable results.

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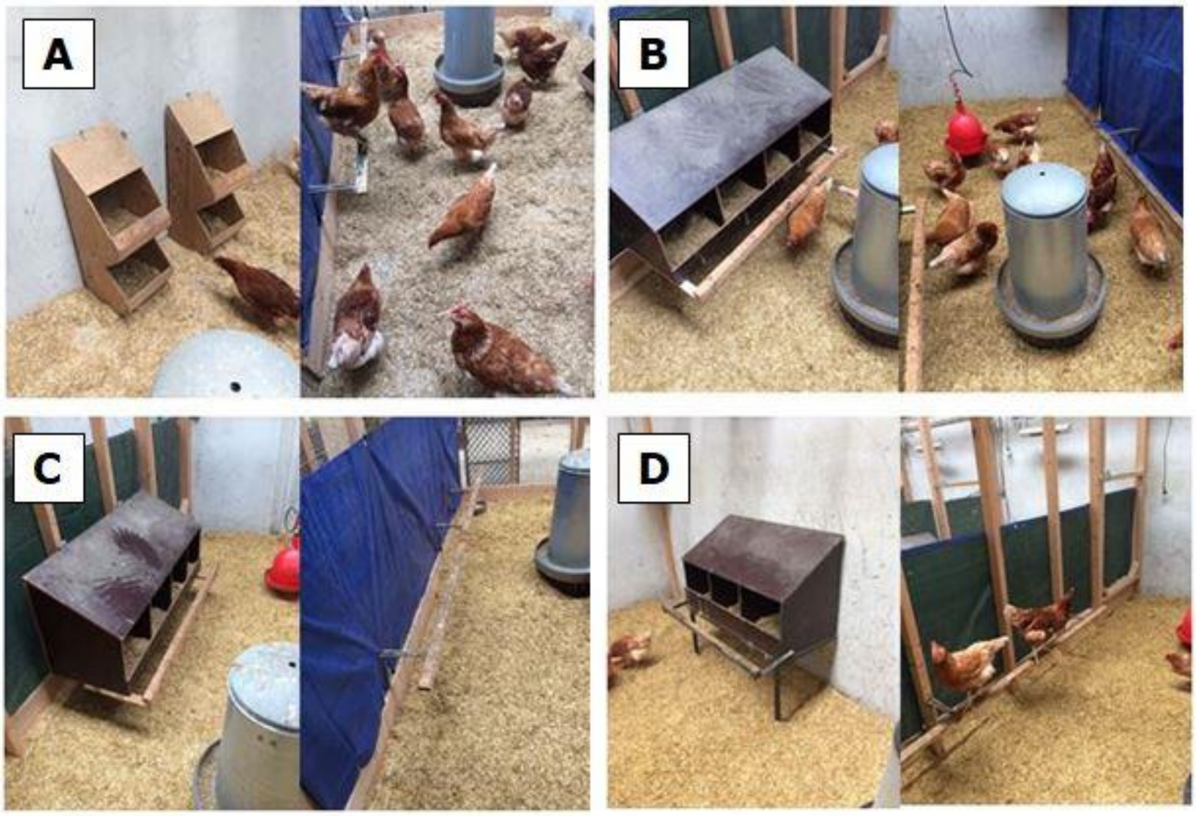
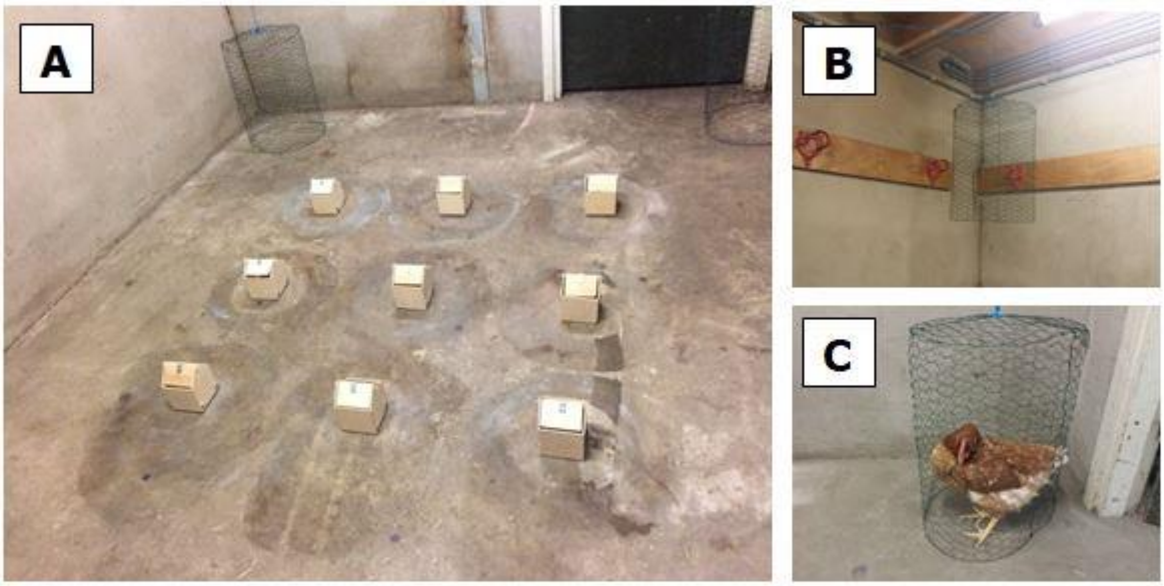
Appendix

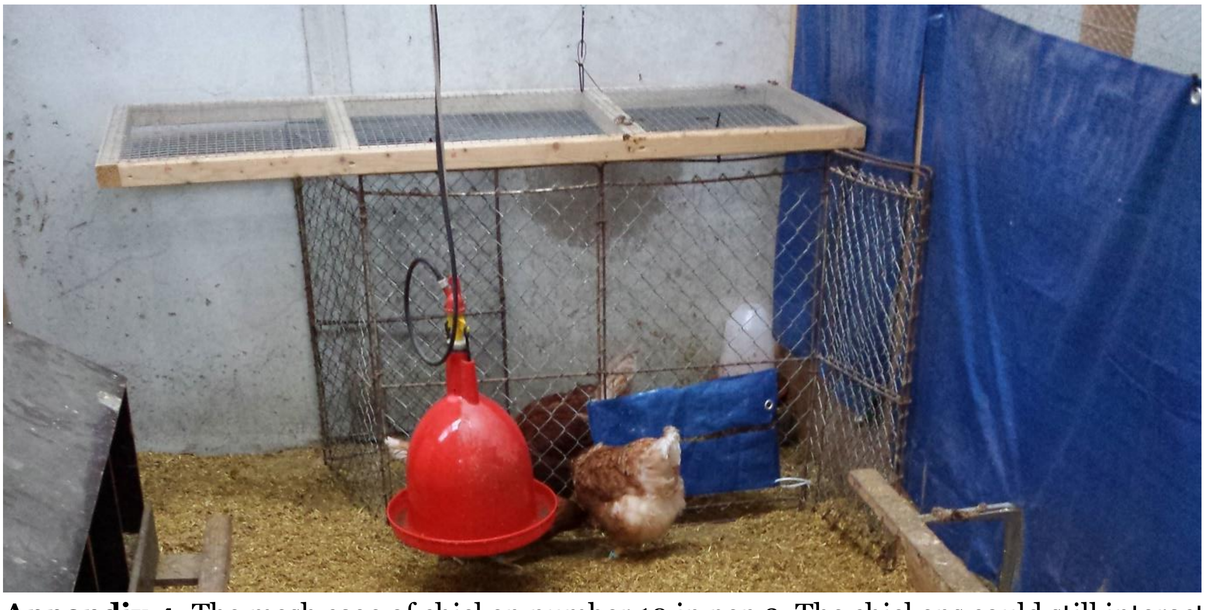


Appendix 1. Home pens. The nest boxes and canvas are shown. Pen 1 consisted of 15 laying hens (panel A), pen 2 consisted of 14 laying hens (panel B) and pen 3 and 4 consisted of 5 laying hens (panel C and D respectively).



Appendix 2. The holeboard room with the renewed cups and starting cages (A). A mesh starting cage (B). A laying hen in a starting cage (C).

Appendix 3. Screen view of a habituation session. One starting cage and one baited cup were used for these sessions.



Appendix 4. The mesh cage of chicken number 10 in pen 3. The chickens could still interact with each other.