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Faculty of Veterinary Medicine and Animal Sciences

The effect of early environment on the adaptability of chicks

−Exploring cognitive aspects

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Department of Animal Environment and Health

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Abstract

Due to the many challenges that laying hens face throughout their lives in the production system, raising hens with the ability to adapt and cope within their environment is vital from both a welfare and production standpoint. The early environment of chicks has been shown to have lasting effects on the health and well being of laying hens later in life. However, few studies have investigated the effects of early environment and adaptability. This study investigated cognitive functions and learning capabilities, which are pivotal in developing adaptation skills, of 48 laying hens at the age of 9-14 weeks using a holeboard test. Hens were raised in differing environments over two periods of rearing: early rearing from 0-4 weeks of age and current rearing from 5-15 weeks of age. The treatments consisted of choice of substrate and perch (four types of each) vs. no choice of substrate or perch (i.e. one type of each), which was changed between the two rearing periods for half of the birds, resulting in four different treatments i.e. choice/choice (CC), choice/no-choice (CN), no-choice/no-choice (NN), and no-choice/choice (NC). When habituating individually to the holeboard test, birds from treatment CC found more worms than those from treatments NN and CN (*p*=0.004; *p*=0.03). During acquisition, a significantly higher number of birds from CC and NC completed the start trials when compared to NN and CN birds (*p*=0.04). Furthermore, results from the reversal phase indicated that NC birds had a higher reference memory than NN birds. Overall these results suggest that having choice in the current rearing environment influenced the birds' success in the holeboard test, which may in turn be directly related the their ability to adapt to new environments and circumstances. Supplementary studies into the critical stage of early rearing and when choice is best suited to be introduced may provide further insights into the role that choice plays on the adaptability of laying hens.

1. Introduction

Chicken and egg production is a vital part of global agriculture with over 23 billion chickens in the world, according to FAOSTAT (2020), and this number expecting to increase along with the global human population. Considering the immensity of the chicken population, providing these animals with the environment and experiences needed to prepare them for a life in production systems is important, not only for the animals themselves, but also for the efficiency and effectiveness of the agricultural industry as a whole. Regardless of the type of production system, birds will face daily challenges to their health and well being, which can only be met if the birds are adequately prepared for what they will face. One particular challenge in poultry production is in understanding the role that early life experience plays in the future adaptability of the birds in question. Through investigating rearing in the early life of laying hens, better understanding of its impact on hen welfare, coping strategies, cognitive function and therefore adaptability may be achieved.

1.1. Red jungle fowl vs. laying hens

While domestication and breeding efforts of laying hens has led to increased productivity and efficiency, it has also resulted in drawbacks in some aspects. A study conducted by Väisänen and Jensen (2004) observed that when young red jungle fowl and White Leghorn layers were given the choice between interacting with familiar or unfamiliar social companions, the Leghorns showed a stronger avoidance to those unfamiliar to them. The authors suggested that this could indicate a reduced ability to cope in stressful situations, particularly those of a social nature. Furthermore, the Leghorns were also found to express more aggressive and confrontational behaviours to conspecifics than observed in Jungle Fowl (Väisänen and Jensen, 2004). A similar study comparing red jungle fowl and Leghorns found that in a stressful environment created by an unpredictable light schedule, red jungle fowl seemed to have better coping capabilities (Lindqvist and Jensen, 2009). Additionally, while stress impaired both breeds' spatial learning ability, Leghorns were more notably affected. The differences seen between the red jungle fowl and Leghorns of these studies indicate that overall, the coping strategy and adaptability of the modern day layer has been reduced when compared to its wild counterpart. Investigating how the adaptability of laying hens might be improved is thus needed, in order to promote better welfare and production potential.

1.2. Adaptability and cognition

The word "adaptability" amongst animals is typically used in referring to their physiological state and their bodies' abilities to acclimate to their surroundings (Bocquier and González-García, 2010; Verbeek et al., 2012). However, adaptability can also be used in terms of "…the cognitive, behavioural, and emotional regulation assisting individuals to effectively respond to change, uncertainty, and novelty." as stated by Burns and Martin (2014) and Martin (2012). This, of course, is an important distinction to consider when discussing this particular term.

Keeping with this definition, more research has begun focusing on how to better prepare production animals, in this case laying hens, to be able to respond appropriately to environmental changes throughout their lives. In particular, the development of spatial cognition is a crucial skill for the laying hen, as it enables them to maximize the use of their resources by locating food, water, perches, and nest boxes. A study by Campbell et al. (2018) found that outdoor-preferring hens had improved spatial cognition in a T-maze test when compared to hens that never went outside. Another study by Tahamtani et al. (2015) investigated the relationship between environmental complexity and various forms of working memory, which can be used to assess spatial cognitive abilities, in laying hens through the use of a holeboard test. The authors determined that birds reared in aviary systems were more adept at solving the holeboard task and demonstrated better working memory than birds reared in a barren environment. These results suggest that a complex environment is important for improved adaptability and cognition in laying hens. Another way of thinking about a complex environment might be to suggest that the birds are given more *choice*, and that this access to choice is pivotal in developmental processes which allow them more success at adapting to new experiences and future environments.

1.3. Effects of rearing period and environment

Numerous studies have investigated the effects that early environment and rearing period have on laying hen welfare and health later on in life. Research has shown that hens are most successful throughout their adult years if they are able to remain in environmental systems similar to those they have been raised in (Widowski and Torrey, 2018). A review by Janczak and Riber (2015) outlines an array of studies where laying hens that were transferred to environments more complex than that of their rearing environments experienced issues such as higher instances of feather pecking, lack of ability to navigate 3-dimensional spaces i.e. perches and nest boxes, increased floor eggs and increased fearfulness. Alternatively, laying hens that were transferred to less complex environments experienced frustration, which resulted in feather pecking and reduced plumage quality, among other things (Janczak and Riber, 2015). Such studies clearly highlight the large role that rearing environment can play on a variety of aspects in the adult life of a laying hen. And while the complexity of the environment as a whole is important to consider during the rearing period, attention should also be paid to the specific aspects of the rearing environment that create this complexity, particularly those aspects of the physical environment which are already acknowledged as being important to the laying hen, such as substrate and perches.

1.3.1. Substrate

Laying hens are motivated to perform certain substrate related behaviours such as foraging and dust bathing, both of which are considered behavioural needs (Weeks and Nicol, 2006; Campbell et al., 2019). In many cases, the ability to perform these behaviours will directly affect the welfare of a hen. As a result, the effect that early access to substrate has on laying hens later in life has been studied at length. Such studies have found that pecking behaviour is especially affected by early access to substrate and inadequate early access to this resource has been shown to be a large factor in instances of feather pecking in adult birds. A study investigating a variety of farm factors and their correlation to feather pecking found that birds raised without access to substrate during the first four weeks of rearing had significantly more feather damage during the laying period (Bestman et al., 2009). Alternatively, birds that did not have feather damage during rearing continued to display good plumage during the laying period, highlighting the importance of early rearing environment and later life effects. Other positive results from early access to substrate that have been observed within adult laying hens include reduced fearfulness, lower mortality, increased plumage quality, increased egg weight and increased foraging and ground pecking (Johnsen et al., 1998; Nicol et al., 2001; Aerni et al., 2005; Janczak and Riber, 2015). While some substrates are superior depending on the purpose of their use, straw, sand, peat and wood shavings are among substrates that have demonstrated positive effects during rearing as previously described, especially when compared to birds raised on wire or mesh (Nørgaard-Nielsen et al., 1993; Huber-Eicher and Wechsler, 1998; Nicol et al., 2001; Nicol et al., 2009). But while the positive effects of individual substrates have been studied at length, few studies, if any, have investigated how early access to several of these substrates or in other words, how having a “choice of substrates”, affects the life of an adult laying hen. Such studies may be valuable in continuing to learn how to best meet the needs of these birds, further advancing welfare standards in laying hen production systems and helping to promote positive bird development.

1.3.2. Perches

Along with substrate, early access to perches has demonstrated positive effects for the adult laying hen. A study conducted by Hester et al. (2013) investigated how access to perches at different life stages affected the musculoskeletal health of White Leghorn hens. The study found that birds that had access to perches as pullets (from hatch to approximately 17 weeks of age) had both improved muscle development and improved bone mineralization as adults, a result that supports previous findings of a similar study (Enneking et al., 2012). Another study conducted by Gunnarsson et al. (2000) observed impaired spatial cognitive skills in laying hens that were not given access to perches early on in rearing, an important finding, especially for those birds in production systems such as aviaries, where 3-dimensional spatial navigating skills are crucial. This can be put into perspective through a study conducted by Ali et al. (2019), where late introduction of perches resulted in less vertical movement by the birds and more frequent and forceful falls at night. Among those already listed, other benefits of early access to perches that have been observed in studies include reduced feather pecking, lower prevalence of floor eggs, and lower occurrences of cloacal cannibalism (Gunnarsson, 1999; Huber-Eicher and Audige, 1999). Much like substrate, there is little to no information on how having access to several different types of perches in early rearing environments affects laying hens later in life. It is therefore of interest if having early choice of both perches and substrate will provide laying hens with the tools necessary in adult life to adapt to a wider variety of environments and situations.

Though it has been touched on briefly, it is important to underline the role that environmental complexity in early rearing stages may play in developing cognition and increasing adaptability (Widowski and Torrey, 2018). However, to what extent is not entirely known, nor is it known exactly at what critical stage of early rearing it is best to target the development of particular cognitive and adaptability based traits. While studies have looked at early rearing and its later affects on bone and overall health, behaviour, resource use, and welfare (Widowski and Torrey, 2018), further information is needed into the learning and cognitive aspects of the early rearing period on laying hens in order to truly understand the effect of the early rearing environment on adult hen adaptability.

1.4. Swedish and the European Union rearing standards

Current industry standards for rearing requirements of laying hens in Sweden have no specific laws regarding environmental complexity and early rearing. However, there is legislation addressing the minimum standard of specific aspects of the rearing environment, such as substrate and perch requirements. According to the States Agricultural Work Statue/ Statens jordbruksverks föreskrifter och allmänna råd om fjäderfähållning inom lantbruket m.m. (SJVFS 2019:23) young hens reared in cages are required a minimum perch space per animal ranging between 20 mm to 100 mm, depending on age (0-18 weeks old), with the same requirements set for free range birds, though 120 mm of perch space per animal must be given between the ages of 17-21 weeks. A minimum of one-third of the ground area of substrate must be provided for young free range hens, while substrate specifications for young caged hens adheres to the general guidelines that there must always be enough substrate for hens to satisfy their behavioural needs such as pecking, scratching, and dust bathing. Apart from the direction that course substrate should not be used in caged systems and perches must be free from sharp edges, no stipulations are in place regarding perch type or substrate type.

From a broader context, the European Union legislation outlined in the Council Directive 1999/74/EC (1999) on laying down minimum standards for the protection of laying hens, that there must be at least 15 cm of perch available per hen when rearing in an enriched cage system. Perches in alternative systems must follow this minimum guideline as well, along with some specific spacing and location requirements. Litter/substrate must also be provided in enriched cage systems, so that pecking and scratching is possible, though a specific amount is not indicated. Alternative systems require that at least one-third of the ground surface contain litter (at least 250 cm^2 of littered area per hen). It is also important to note that while these provisions are required for laying hens that have reached maturity, specific legislation is not in place for chicks or pullets throughout early rearing, apart from the general directives laid out in the Council Directive 98/58/EC (1998) concerning the protection of animals kept for farming purposes. In order to better support the adaptability of laying hens, more specific legislation regarding environmental complexity may be needed. However, before this can occur, investigation into what specific aspects of early rearing and environmental complexity affect the adaptability and coping skills of laying hens is needed.

1.5. Holeboard test

The holeboard test is a behavioural test that has been used in numerous studies to assess memory, spatial learning and cognitive abilities of a variety of species such as rodents, pigs and poultry, among others (Nordquist et al., 2011; Subramaniyan et al., 2015; Grimberg-Henrici et al., 2016). While there are many different variations of the holeboard test, it typically consists of an acquisition phase and a reversal phase. During the acquisition phase, a series of cups are set up throughout the holeboard arena and the animal is taught to find a specific set of these cups that are baited, while the rest of the cups remain empty. Once this phase is complete, the animal moves forward to the reversal stage of the holeboard test. Here it follows the same procedure as the acquisition phase, though the previously baited cups remain empty and a series of different cups are baited.

One particular benefit of the holeboard test is the ability to measure several variables at once, both cognitive based and non-cognitive based. These variables may include, but are not limited to, habituation processes, reference memory, working memory, general working memory, exploration, and anxiety-related behaviours (van der Staay et al., 2012). Working memory ratios represent the ability of the animal to avoid revisiting a reward/baited cup after already finding the mealworm in that cup, while general working memory ratios represent the ability of the animal to avoid revisiting cups, including both reward cups and empty cups (van der Staay et al., 2012; Tahamtani et al., 2015). In other words, working memory represent the animal's ability to remember having already found the reward from a specific baited cup, while general working memory represents the animal's ability to remember which cups in the holeboard it has already visited (and therefore maximize its time in searching cups it hasn't visited yet). Reference memory ratio represents the ability of the animal to learn and remember which cups are baited and which cups are not (van der Staay et al., 2012; Tahamtani et al., 2015). An animal with a high reference memory ratio will waste less valuable time searching empty cups, and will instead focus on those cups that are baited.

The aim of this study was to investigate the effect that the early versus the later rearing environment has on the adaptability of laying hens through the use of a holeboard test. Specifically, four treatment groups were subjected to either choice of different substrates and perch types, no-choice of substrate or perch types, or a combination of the two (changing treatment between the two rearing periods). A holeboard test was then conducted in order to study the spatial learning, memory and overall, the adaptability of birds from the various treatment groups. It was hypothesized that birds receiving the treatment of choice throughout the entire rearing period, and birds that only had choices when in the early rearing environment would be more successful in the holeboard test compared to those birds that received the no-choice treatment throughout the entire rearing period or during the current rearing environment, due to increased cognitive abilities and adaptability fostered by the choice environment.

2. Materials and methods

2.1. Ethics

All tests throughout the study were performed in compliance with the ethical application 5.8.18 11549/2017.

2.2. Animals and housing

This study used 48 Bovan Robusta laying hens housed at the Swedish Livestock Research Centre, Lövsta, in Uppsala, Sweden. The birds were a subset of a larger group of 364 birds housed in 16 pens, where 22-23 birds were kept per pen. The hens were day old to 14 weeks of age over the course of the study period and participated in holeboard testing from 9-14 weeks of age. All pens housing the birds included appropriate water drinkers and feeders. Lövsta staff routinely cared for the birds following normal barn procedures and the recommended lighting schedule.

2.3. Treatment

Rearing of the hens in this study was separated into two stages: early rearing environment (0-4 weeks of age) and later rearing period (5-15 weeks of age)(Fig. 1). Two types of pen environments were used throughout the rearing periods of the birds: choice pens (C) and no-choice pens (N). This allowed for the hens to be divided into four different environmental treatment groups over the course of the two rearing periods (Fig. 2); choice/choice (CC), choice/no-choice (CN), no-choice/no-choice (NN) and no-choice/choice (NC). As 16 pens of birds were used within this study, this resulted in the early rearing environment consisting of eight pens of choice birds, and eight pens of no-choice birds. When the later rearing environment (which was the current environment at the time of testing) began at 5 weeks of age, 4 pens from each treatment continued to receive the same rearing environment, while 4 received the alternate rearing environment.

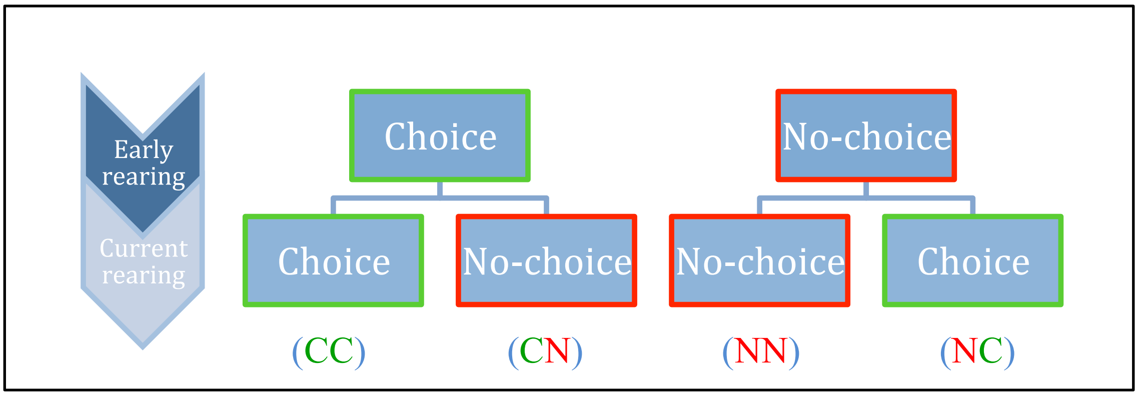

Fig. 1. Schematic representation of treatment plan over early and current rearing environments.

Fig. 2. Pen overview of choice (top row) and no-choice (bottom row) treatments during the early rearing environment. (a) Choice pens with different ground substrates, access to all four litter types (straw, wood shavings, sand and peat provided in trays) and all four perch types (wire, rope, round rubber, and solid wood) and (b) no-choice pens with one type of substrate and one type of perch. *Photo by Lena Skånberg*.

In each home pen, four trays were placed on the floor and covered approximately two-thirds of the pen. Each tray was filled with a particular substrate, depending on the treatment. In the choice pens, one of each tray was filled with peat, sand, wood shavings and straw. Choice pens were also given access to four perches: a braided rope, a mesh platform, a wooden platform and a rubber bar. Hens that were raised in the no-choice pens had access to only one of the aforementioned substrates, which filled all four trays. While no-choice pens were also fitted with four perches, only one perch type was used. The substrates, perches, and order of the placement of both substrate and perches in the pens were balanced across treatment groups. In the early rearing environment substrate was spread across the bottom of the pen to ensure chicks could not escape through a gap between the pen wall and the floor and to provide extra warmth against the concrete. For choice pens, the floor substrate was chosen randomly and balanced across choice treatments, while no-choice pens received the same substrate as already within the trays. The outside of all pens used within the study was wrapped with brown paper to prevent birds from being affected by surrounding pens.

2.4. Holeboard test

Three birds from each home pen were chosen at random for holeboard testing based on their location within the pen. One bird was chosen from each of the following three locations: front of the pen or on top of any perch inside the pen, middle of the pen, and back of the pen. Birds were chosen in this manner to avoid catching only birds close to the catcher that may be less fearful, therefore creating a personality bias of the test birds. As there were four pens of each type of treatment, a total of 12 birds from each treatment participated in the holeboard test. Once chosen, the birds were then fitted with an extra leg ring to allow for easy identification of test participants. Before both habituation and holeboard testing, the birds were caught and placed inside a spacious holding box with the other two test birds from their home pen. While one bird was being tested in the arena, the other two remained inside the box to await testing. Birds were kept in the holding box for no longer than 30 minutes before being tested. All birds were habituated or tested throughout various times of the day over the course of the study to control for any effects time of day may have had. If any birds were omitted from the study due to not reaching the testing criteria, they continued to be caught and placed in the waiting box while pen mates were being tested. This was to ensure the environment and experience of the holeboard habituation and testing was the same for birds in all pens. For a complete timeline of the habituation and holeboard test, as well as how many birds participated in each phase of the test, refer to Table 1.

Table 1. Habituation and the holeboard test timeline, including trials, type (whether the bird was in the arena in a group or alone), and the total number of birds that participated in each phase of the holeboard test.

	Habituation				Acquisition			Reversal
Trial #	1-3	4	5	6	1-4 (Start trials)	5-10 (Middle trials)	11-21 (End trials)	22-31
Type	Group	Alone	Group	Alone	Alone	Alone	Alone	Alone
# of birds in test	48	48	48	48	21	17	8	4

2.4.1. Holeboard arena

The holeboard arena was constructed using wire compost gates covered in brown paper that the birds were already familiar with and measured 248 cm x 218 cm (Fig. 3). Nine white cups, each with a diameter of 6 cm and a height of 5 cm, were placed inside and

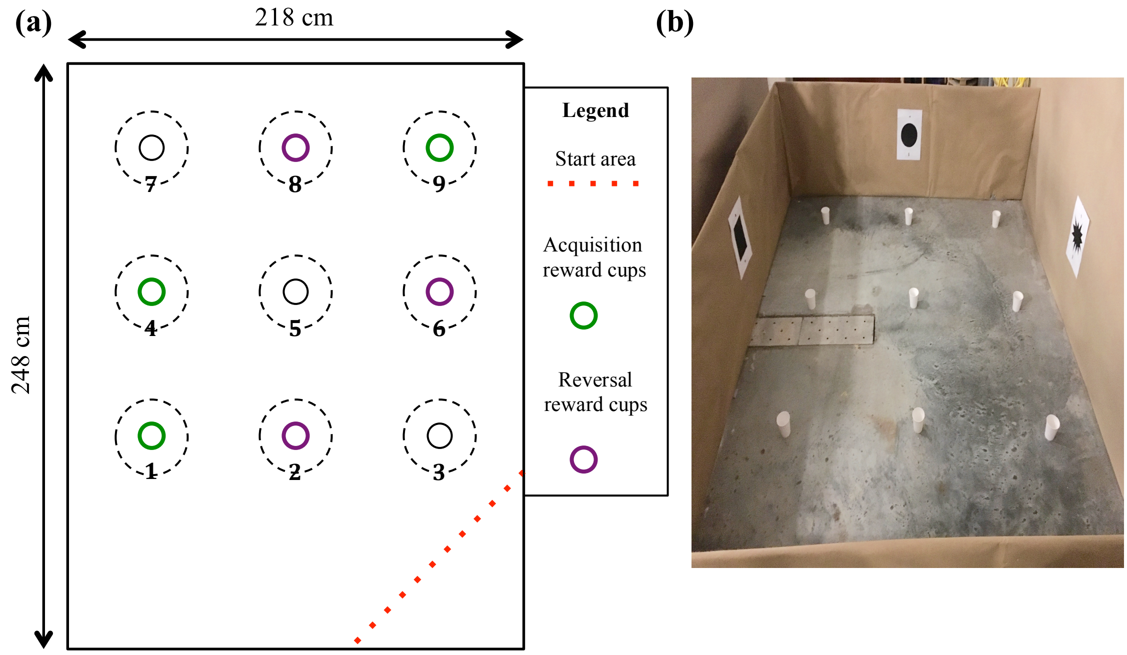

Fig. 3. (a) Schematic representation and dimensions of the holeboard arena and (b) photo of the holeboard arena used in testing.

glued to the floor where they were used to offer a reward/bait of a live mealworm. These cups were spaced 70 cm apart from one another (30 cm from arena wall) and when baited, only one mealworm was used per reward cup. Circles 40 cm in diameter were drawn on the concrete floor of the arena around each cup using white chalk. When a bird placed both feet entirely in a circle, or when one foot was placed inside the circle and the neck was stretched out toward the cup, the cup was considered “chosen” by the bird. The front area of the arena by the starting corner was 50 cm from the nearest row of cups to make it obvious when the birds began the test by exploring the cups. On each side of the arena was a picture of a different shape (star, triangle, square, and circle) in order to allow the birds to be able to orientate themselves while inside the holeboard arena, though other spatial clues were present due to the arena construction, such as the drain in the floor or the concrete wall on one side. The top of the arena was covered in wire mesh to prevent the birds from flying out while inside. A Garmin Virb 360 camera was hung from the ceiling directly above the holeboard arena and was used to film all holeboard test trials.

2.4.2. Habituation

Before beginning the holeboard testing, the chosen three birds from each pen were habituated to the holeboard arena in “habituation sessions” (for simplicity’s sake, when referring to the habituation period, the term “sessions” will be used, while the term “trials” will be used when referring to the holeboard test itself). During the first habituation session, each cup fastened to the floor of the arena was baited with one mealworm. The birds were placed inside the start corner of the holeboard arena with their respective pen mates. They were then allowed to explore the arena for a period of 10 minutes or until all nine of the mealworms were consumed. The arena was cleared of any defecation between all sessions. During the second habituation session, each of the nine cups was placed upside down on the arena floor, and baited with one mealworm on top of each cup. This was done, as very few birds expressed interest in the worms or the cups during the first habituation session. The birds were again habituated in groups of three and were free to explore the holeboard arena for a period of five minutes, or until all nine of the mealworms were consumed. After this habituation session, the birds began to associate the cups with the mealworms and thus, the cups were flipped right side up and baited with a mealworm inside as done previously. Group habituation continued until session number three, after which one session of habituation alone was given (Table 1). Group and individual habituation sessions then alternated until each bird completed a total of six sessions of habituation i.e. four group sessions and two individual sessions. The habituation to the holeboard was the only time that the birds were inside of the arena in groups. In order for birds to move onto the acquisition of the holeboard test a criteria goal was set that they must have eaten at least three mealworms (out of nine) in one or both of their habituation sessions alone. This was to ensure not only that the birds were ready to enter the acquisition phase, but also that they were motivated enough to eat three worms, which would be required of them once reaching the acquisition and reversal phases.

2.4.3. Holeboard test procedure

The holeboard test itself consisted of two phases: the acquisition phase and reversal phase, and was adapted from Nordquist et al. (2011). Within the acquisition phase three different criterion goals were set, which in turn created three distinct periods within the acquisition phase: start trials (trials 1-4) with the participating birds that passed the habituation criterion, middle trials (trials 5-10), and end trials (trials 11-21). Any birds that did not reach the criterion goal (explained below) before the next period were omitted from the remainder of the holeboard test. During the acquisition phase, the birds were tasked with finding three specific cups in the arena that were baited with live mealworms while the other six cups remained empty. The three cups 1, 4, and 9, were assigned as bait cups for all birds throughout the acquisition trials, while cups 2, 6, and 8 were used as bait cups during reversal trials (Fig. 3a). This was done randomly apart from the constraint that there had to be one bait cup in each of the horizontal rows of the test, and that reversal cups needed to differ from acquisition cups. The criterion goals to pass each period of the acquisition phase were as follows:

First criterion (to pass start trials): birds were required to find a total of at least four worms throughout the start trials, which equated to an average of one worm per trial.

Second criterion (to pass middle trials): birds were required to find at least six worms within the last three trials, which equated to an average of two worms per trial.

Third criterion (to pass end trials and begin reversal phase): birds were required to find all three worms in a single trial at least one time within the last three trials. The criterion was set in this way to ensure that the birds had successfully learned the baited cups of the acquisition before continuing on to the reversal phase.

While completing the holeboard test, each bird was tested individually and time between consecutive trials for one bird was between 5-20 minutes. A maximum of five tests a day were conducted for each bird, though this only occurred during the reversal phase of the holeboard test, as less birds were participating. Before a trial began, the test bird was gently lifted out of the holding box and placed into the arena in the starting corner. The trial started as soon as the wire roof was resecured and was concluded after the bird successfully found the worms in all three baited cups, or once the test duration reached a maximum time of five minutes. The holeboard arena was cleaned of any defecation or debris from the previous bird between each test.

2.4.4. Holeboard variables

Latency to first cup, latency to first worm, worms found, and test duration were recorded for habituation sessions and holeboard trials. During holeboard trials, reward cup visits, cups revisited, and total cups visited were also recorded. From these observations, working memory, general working memory and reference memory ratios were calculated for the acquisition and reversal phases. For a complete description of all variables and definitions see Table 2.

Table 2. Ethogram of holeboard variables.

Variable	Definition
Cup latency (latency to first cup)	The time taken from the moment the bird was placed into the arena (start of test) to the choice of first cup. A cup was chosen when the bird had both feet inside the chalk circle surrounding the cup, or when one foot was inside the circle and the neck was extended toward the cup.
Worm latency (latency to first worm)	The time taken from the start of the test to when the first mealworm was found. The mealworm was considered found when the bird interacted with it in any way by touching it, or when the mealworm was eaten.
Worms found	The number of mealworms found i.e. interacted with and/or eaten during the test.
Test duration	The duration of the test beginning from the moment the bird was placed in the arena to the time taken to find all three mealworms or for the maximum amount of time to occur i.e. 5 minutes.
Cups visited	The number of individual cups that were chosen by the bird (out of 9).
Total reward cups visited	The total number of reward cups that were chosen in the test, including revisits.
Total number of cups visited	The total number of cups that were chosen in the test, including revisits.
Working memory	The number of rewarded visits i.e. worms found, divided by the number of total reward cups visited.
General working memory	The number of cups visited (out of 9) divided by the total number of cups visited (includes revisits).
Reference memory	The number of total reward cups visited divided by the total number of cups visited.

2.5. Statistical analysis

All data was analyzed using the statistical program R and R Studio. The variables cup latency, worm latency, test duration, and worms found were analyzed for habituation and the two phases of the holeboard experiment (i.e. acquisition, reversal). Additionally, working memory, general working memory, and reference memory ratios were investigated for acquisition and reversal phases of the holeboard test. Early rearing environment and current rearing environment were included separately within the model, as this allowed for the investigation as to which rearing environment (or an interaction of both) the choice or no-choice of substrate and perches had the greatest effect. As a hypothesis driven approach of analysis was used, only significance results of interest were analyzed using pairwise comparisons or the appropriate post-hoc analysis for the statistical test used.

Linear mixed models (LMM) were used when analyzing normally distributed data of variables such as latencies and proportions. Values for these variables were logged if improved normality and homoscedasticity were found. Generalized linear mixed models (GMM) were used to analyze variables with counted data, such as number of worms found. Data that were not normally distributed were analyzed using the non-parametric tests Kruskal Wallis or Friedman. Estimated marginal means are presented from mixed models. For data that were log transformed, backtransformed estimated marginal means are then used.

2.5.1. Habituation analysis

Latency to first cup and latency to first worm were analyzed using a LMM. As these data were not normally distributed, the log values of the data were used during analysis. Results and values in graphs were then backtransformed. Means for all individual birds were calculated for each test variable. Within the model the early rearing environment, current rearing environment, and type of habituation i.e. alone or with pen mates (group) were considered as fixed effects and were tested for interactions. Chick was included in the model as a random effect, though pen and trial were not, as this created a model that failed to converge. Time of day was not included in the model, as this was already balanced across treatments. Worms found was analyzed similarly, though a GLMM was used.

Test duration was not normally distributed and log values were ineffective at transforming the data. Therefore, this variable was analyzed using the non-parametric Kruskal Wallis test. Means per pen across all sessions were calculated and used during analysis.

2.5.2. Holeboard test– acquisition phase analysis

For an overview of the holeboard test, the number of birds within each treatment group to successfully pass each of the three periods of the acquisition (start trials, middle trials, and end trials), as well as the habituation, was analyzed using a chi-squared test. As birds had been omitted throughout the acquisition, each period of the acquisition had to be analyzed separately. Analysis for the remaining holeboard variables was only completed for the start trials of acquisition (trials 1-4) and the end trials of acquisition (trials 11-21). The middle trials of acquisition (trials 5-10) were not analyzed, as it was the initial and end stages of learning that were most relevant when exploring memory ratios. This also allowed for a reduction in the overall number of tests performed. In order to better understand if birds omitted were performing poorly due to difficulties learning the test or other factors, the working memory of all birds (as well as by treatments) remaining in the test (“in”) and birds omitted from the test (“out”) were analyzed for each of the three periods of the acquisition (start trials, middle trials, end trials) using a LMM. Working memory was chosen to analyze in this regard as it is the most representative of how the birds learned in the test from a short-term perspective, and is least susceptible of being skewed due to the birds not completing the test properly i.e. not exploring. Working memory ratios were calculated by dividing the number of rewarded visits i.e. worms found by the number of total reward cups visited, which included revisits.

The variables cup latency and worm latency were analyzed using a LMM. Within the model used for the start trials of acquisition, the early rearing environment and current rearing environment were considered as fixed effects and were tested for interaction. Pen, chick, and trial were included in the model as random effects. When analyzing the end trials of acquisition, treatment was considered to be a fixed effect rather than early and current rearing environments. This was done, as there were no birds in one of the treatment groups in the end trials of acquisition. While pen, chick and trial were considered as random effects for worm latency, only pen and chick were included in the model for cup latency, as the model could not converge otherwise.

Test duration for the start trials of acquisition, worms found in the start trials of acquisition, and memory ratios were analyzed using the non-parametric Friedman test, as the data were not normally distributed and could not be transformed. Means of treatment groups (CC, NC, CN, NN) across trials were calculated for each variable and used during analysis. Post-hoc analysis using the Nemenyi test was completed for worms found in both the start and end trials of the acquisition phase. Test duration for the end trials of the acquisition phase was analyzed using a linear mixed effects regression, with the same model used for worm latency, data were log transformed in order to achieve normality. Worms found in end trials of the acquisition phase were analyzed using descriptive statistics due to the small sample size of birds remaining in the test.

2.5.3. Holeboard test- reversal phase analysis
All holeboard test variables in the reversal were analyzed using descriptive statistics, due to the small sample size of birds remaining in the test.

3. Results

All significant results from the holeboard test are summarized in Table 3. Significant results of particular importance are represented in graphs. A summary of means of all habituation and holeboard test variables for each of the four treatment groups can be found in the appendix in section 9.1., 9.2., and 9.3.

Table 3. Summary of significant results and trends of variables of treatment groups (CC, NC, CN, NN) within the habituation, which includes the group and alone sessions, and within the holeboard test phases, which includes trials 1-4 of the acquisition (start trials), trials 11-21 of the acquisition (end trials), and trials 22-31 within the reversal phase.

Variable	Session	Significant effect	Test statistics	Pairwise comparisons where P < 0.10
Worms found	Habituation	Early*Alone/Group Current* Alone/Group	x^2=5.83, df=1, P=0.02 x^2=10.79, df=1, P=0.001	In Alone testings: CC **more** than CN (P=0.003) CC **more** than NN (P=0.03) NC **more** than CN (P=0.07)
	Acquisition – Start trials	Treatment|Trial	x^2=8.14, df=3, P=0.04	CC **more** than CN (P=0.07)
	Acquisition – End trials	Treatment|Trial	x^2=13.00, df=2, P=0.002	CC **more** than NN (P=0.08)
# of birds passing criterion	Acquisition – Start trials	Treatment	x^2=8.18, df=3, P=0.04	CC & NC > NN > CN
Cup latency	Habituation	Current*Alone/Group	$F_{1, 236.0}$= 3.54, P=0.06	In Alone/Group testings: CN & NN alone **slower** than CN & NN group (P=0.02) In Alone testings: CN & NN alone **slower** than NC & CC alone (P=0.07)
Worm latency	Habituation	Current*Alone/Group	$F_{1, 236.0}$= 8.72, P=0.003	In Alone/Group testings: CN & NN alone **slower** than CN & NN group (P=0.003) In Alone testings: CN & NN alone **slower** than NC & CC alone (P=0.02)
Test duration	Habituation	Current*Group	x^2=4.87, df=1, P=0.027	In Group testings: CN & NN **slower** than NC & CC (P=0.09)

3.1. Habituation

3.1.1. Worms found

The number of worms found by the birds was significantly affected by interactions between the early rearing environment and type of habituation (x^2= 5.83, df= 1, *p*= 0.02), and the current rearing environment and type of habituation (x^2= 10.79, df=1, *p*= 0.001)(Fig. 4). Further pairwise comparisons revealed that during habituation alone, birds from the treatment CC found a significantly greater mean number of worms than compared to birds from both treatments CN (*p*= 0.004) and NN (*p*= 0.03). A tendency for a difference in the number of worms found during habituation alone was also displayed between treatments NC and CN (*p*= 0.07), where treatment NC tended to find a greater mean number of worms. Pairwise comparisons did not reveal any significant differences on worms found during group habituation.

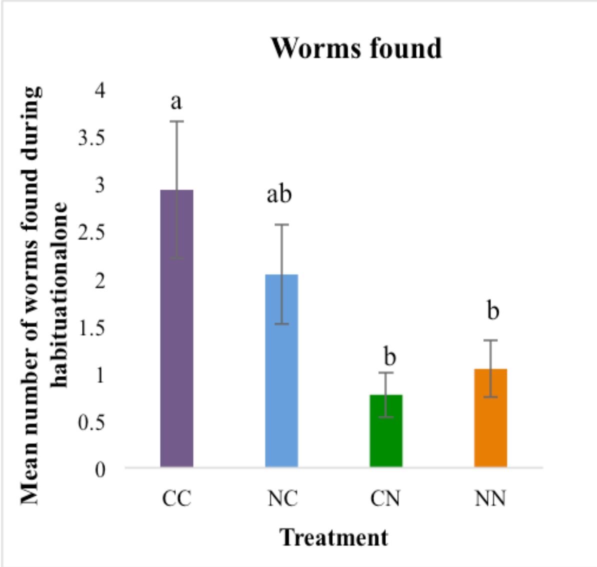

Fig. 4. Mean number of worms found during habituation alone by birds from treatment groups CC, NC, CN and NN. Columns that do not share a letter are significantly different from one another.

3.1.2. Cup latency, worm latency, and test duration

A trend in the main treatment effect for latency to first cup was found in regards to an interaction between the current rearing environment and type of habituation i.e. alone vs. group ($F_{1, 236.0}$= 3.54, *p*= 0.06)(Table 3)(Fig. 5a). Further pairwise comparisons revealed

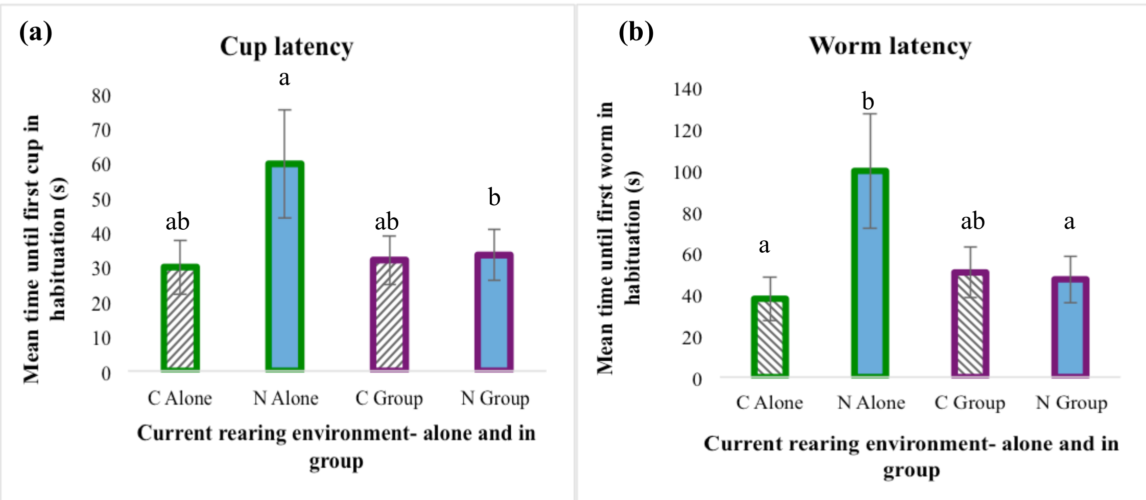

Fig. 5. (a) Mean time to choosing the first cup and (b) mean time to finding the first worm for current rearing treatments while alone and in groups during habituation sessions. “C” refers to treatments CC and NC, while “N” refers to treatments NN and CN. Columns that do not share a letter are significantly different from one another.

that birds in the current rearing environment of no-choice (NN and CN) were significantly slower at reaching the first cup during habituation alone than compared to group habituation (p= 0.02). A trend was also found within habituation alone, which indicated that birds in the current rearing environment of choice (CC and NC) were faster at reaching the first cup than compared to the current rearing environment of no-choice (NN and CN)(p= 0.07).

Similarly to latency to first cup, a significant interaction for latency to first worm was found between the current rearing environment and the type of habituation experienced by the birds ($F_{1, 236.0}$= 8.72, p= 0.003)(Fig. 5b). After pairwise comparisons it was evident that birds in the current rearing environment of no-choice (NN and CN) were significantly slower at finding their first worm during habituation alone than compared to group habituation (p= 0.003). Differences between the birds in the current rearing environment during habituation alone were also found, where choice treatments (CC and NC) were significantly faster at finding their first worm than when compared to no-choice treatments (NN and CN)(p= 0.02).

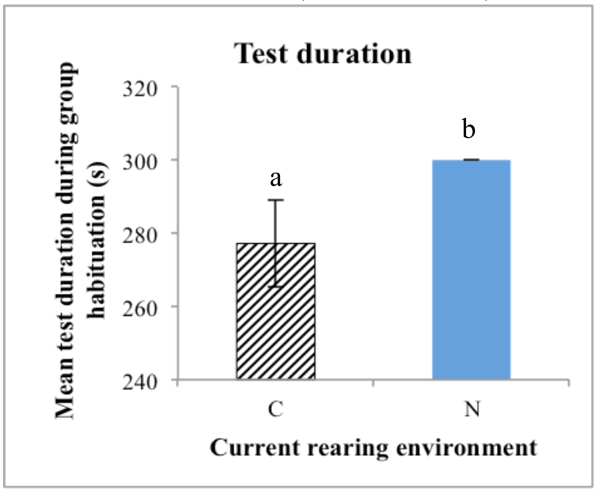

Fig. 6. Mean test duration of the current rearing environment choice and no- choice during group habituation. "C" refers to treatments CC and NC, while "N" refers to treatments NN and CN. Columns that do not share a letter are significantly different from one another.

Current rearing environment was found to have a significant effect on mean test duration during group habituation (x^2= 4.87, df= 1, p= 0.03)(Fig. 6) where birds in the current rearing environment of choice (CC and NC) were faster at completing the habituation sessions than birds in the current rearing environment of no-choice (NN and CN). Test duration showed a strong trend of being affected by treatment during group habituation (x^2= 7.62, df= 3, p= 0.054). The mean test durations for the four treatment groups were as follows: CC= 262.73 ± 21.25, NC= 291.67 ± 8.33, CN=300.00 ± 0.00, NN= 300.00 ± 0.00.

3.2. Holeboard test- acquisition phase

3.2.1. Completion of acquisition periods

A significant effect of treatment was found for the number of birds reaching the criterion to pass the start trials of the

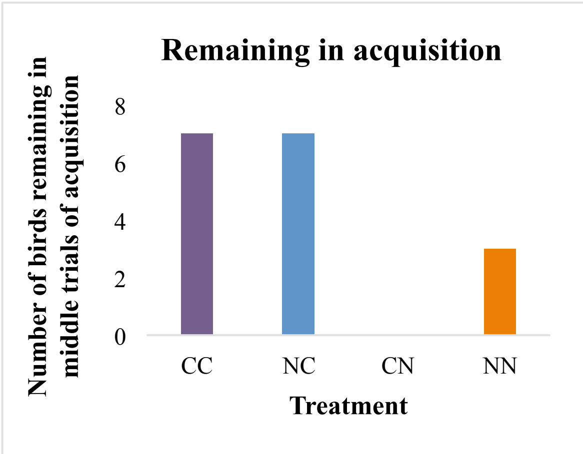

Fig. 7. Number of birds in each treatment (CC, CN, NC, NN) remaining in the acquisition after passing the first criterion to begin middle trials.

holeboard acquisition (x^2= 8.18. df= 3, p= 0.04)(Fig. 7). The number of birds from each treatment that successfully reached the criterion for this period were as follows: CC= 7, NC= 7, CN= 0, NN= 3, whereas no significant effects of treatment were found for reaching the criterion to pass the habituation, middle trials of the acquisition phase, or end trials of the acquisition phase (Table 4). Results from working memory means of birds omitted and birds that remained in each phase of the test are located in appendix 9.2. and 9.3. Birds that remained in the test displayed a significantly higher working memory in the start trials ($F_{1, 19}$= 20.57, p= 0.0002) and middle trials ($F_{1, 15}$= 23.57, p= 0.0002) of the acquisition phase when compared to birds omitted. No significant results were found between the working memory ratios of birds within the end trials of the acquisition phase.

Table 4. Total number of birds taking part in each period of the study i.e. the number of birds from each treatment that successfully **passed** the criterion from the preceding period.

Phase of holeboard test	CC	NC	CN	NN
Acquisition start trials (passed habituation)	7	8	2	4
Acquisition middle trials (passed start trials)	7	7	0	3
Acquisition end trials (passed middle trials)	3	4	0	1
Reversal (passed end trials)	1	2	0	1

3.2.2. Worms found

A significant effect of treatment for worms found by the birds was shown in the start trials (x^2= 8.14, df= 3, p= 0.04) of the acquisition phase, while a possible effect of treatment was displayed for the end trials of acquisition. Post-hoc analysis revealed a trend where CN birds found fewer worms than CC birds (p=0.066) in the start trials of acquisition (Fig. 8). During the end trials of the acquisition phase, CC birds seemed to find fewer worms than the NN bird (Fig. 9). Refer to appendix 9.3., Table 9., for a summary on worms found during start, middle and end trials.

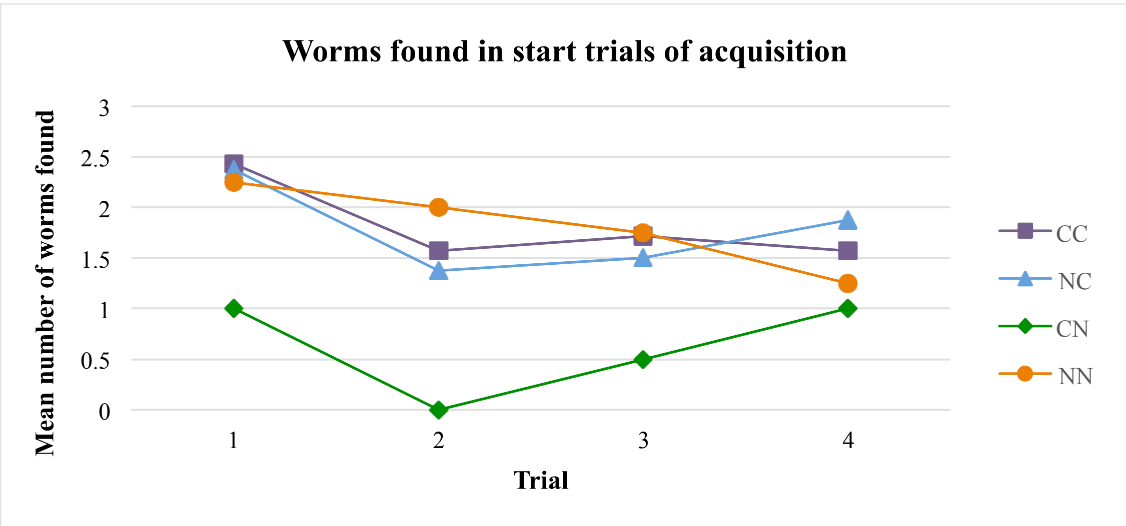

Fig. 8. Mean number of worms found per trial in the start trials of the acquisition phase for treatments choice/choice (CC), no-choice/choice (NC), choice/no-choice (CN), and no-choice/no-choice (NN). Number of birds in each treatment is as follows: CC=7, NC=8, CN=2, NN=4.

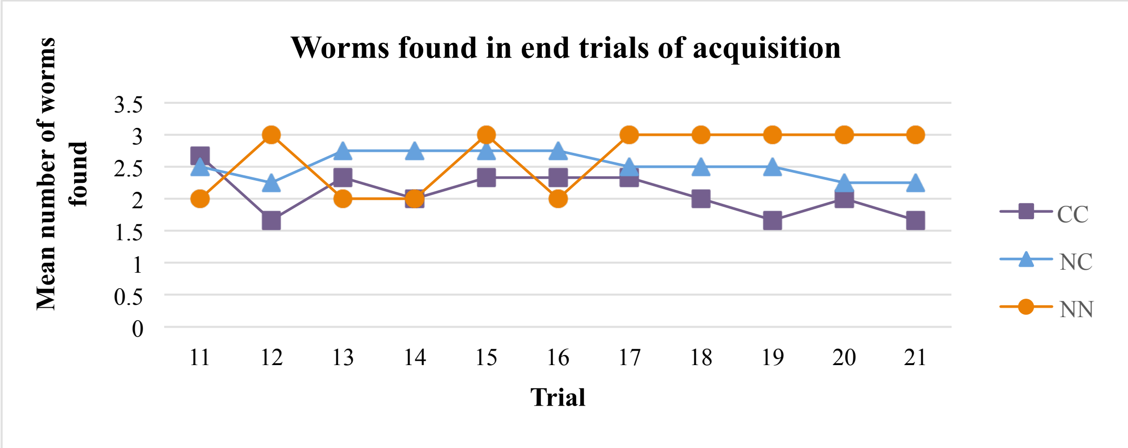

Fig. 9. Mean number of worms found per trial in the end trials of acquisition for treatments choice/choice (CC), no-choice/choice (NC), and no-choice/no-choice (NN). No birds from the choice/no-choice (CN) treatment reached the criterion to be included in these end trials. Number of birds in each treatment is as follows: CC=3, NC=4, NN=1.

3.2.3. Cup latency, worm latency, test duration and memory ratios

No significant effects for early rearing environment or current rearing environment were found for cup latency, worm latency, test duration or memory ratios in either the start trials or the end trials. Refer to appendix 9.1. for a summary of all test variable means.

3.3. Holeboard test- reversal phase

Of the four birds that participated in the reversal phase (CC:1, NC:2, NN:1), no clear differences of treatment was found for worm latency, cup latency, test duration or worms found during the reversal phase of the holeboard test.

3.3.1. Memory ratios

Differences of reference memory ratios were found between treatments where the two NC birds had a higher reference memory ratio than the NN bird and the CC bird (Fig. 10 and Fig. 11). Means and standard errors of the reference memory of each of the four birds from the treatment groups in the reversal are as follows: CC= 0.35 ± 0.02, NC1= 0.41 ± 0.03, NC2= 0.44 ± 0.02, NN= 0.35 ± 0.02. No effects of working memory or general working memory were found.

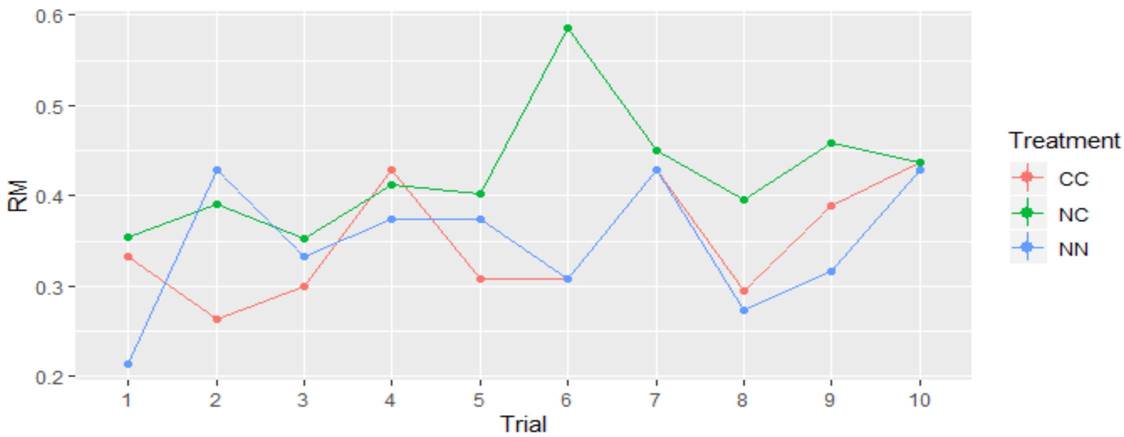

Fig. 10. Mean reference memory (RM) ratio for birds in treatments CC, NC and NN in each trial of the reversal phase. Number of birds in each treatment is as follows: CC=1, NC=2, NN=1. Reference memory was calculated by dividing the total number of reward cups visited, including revisits, by the total amount of cups visited, including revisits.

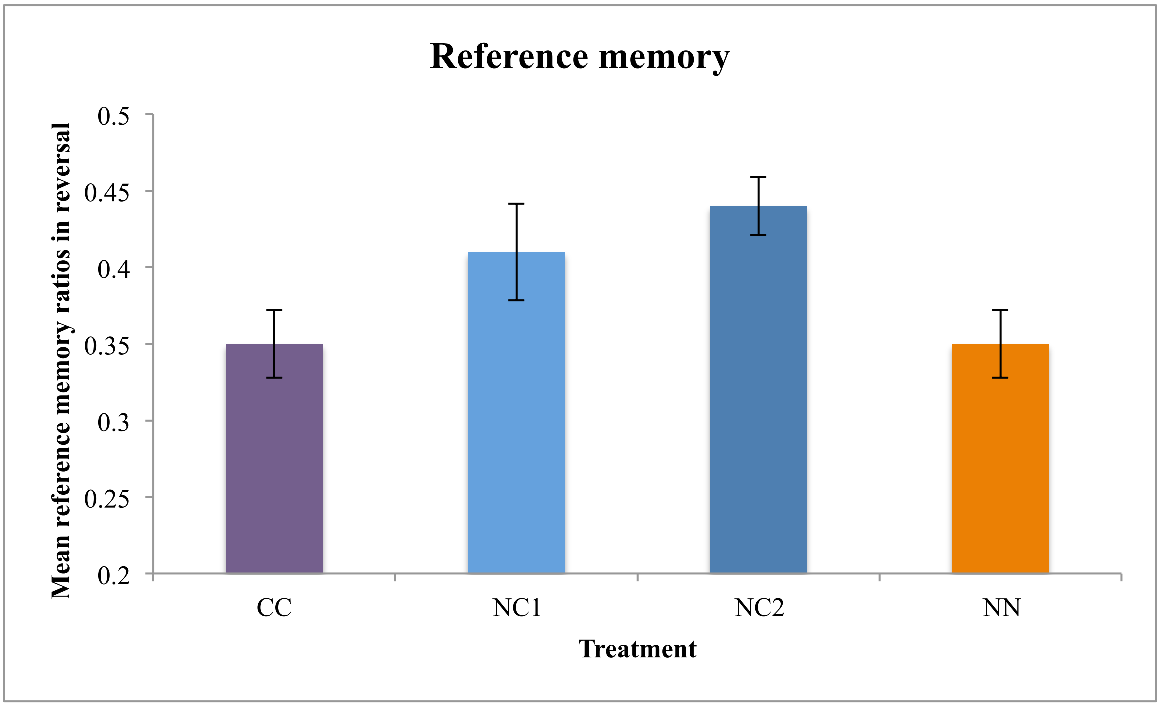

Fig. 11. Mean reference memory ratios of each of the four birds that participated in the reversal phase of the holeboard test (CC=1, NC=2, NN=1).

4. Discussion

In order to raise laying hens with the ability to adapt to the environments and challenges that they will later face, providing an early rearing environment that supports chick development and adaptability is crucial. The results from this study found that providing the treatment of choice during the period of rearing termed "current rearing environment" was largely responsible for affecting bird performance in the holeboard test, and thereby may impact adaptability success. These results support the hypothesis that rearing in environments of complexity in the form of choice is an important factor in developing spatial cognitive skills needed by hens to adapt appropriately to new environments and experiences. However, the hypothesis that the early rearing environment would be the most influential in promoting the development of learning and cognition was not clearly supported.

4.1. Overall treatment effects

From an overview of the habituation and holeboard test, clear differences were seen amongst treatment groups. During habituation, treatments CC and NC were more successful at finding worms when they were alone and completed group habituation sessions faster than the treatments NN and CN. The treatments CC and NC also contained the largest number of birds that passed the start trials criterion in the acquisition, a pattern that continued into the reversal phase of the test. Conversely, CN and NN had low numbers of successful birds throughout the duration of the experiment, with CN being omitted entirely after the start trials of the acquisition. During the reversal trials the birds in treatment NC further excelled, displaying a higher reference memory than the birds in treatment NN. As both CC and NC were exposed to the treatment of choice in the current rearing environment, while CN and NN had no-choice, it seems apparent that the treatment of choice, particularly in the current rearing environment, best prepared the birds for success within the holeboard test. Furthermore, analysis of the working memory of birds remaining in the holeboard test compared to birds omitted in each of the three periods of the acquisition showed that the birds remaining in the start trials and middle trials had significantly higher working memory ratios compared to omitted birds. Means also differed in the end trials of the acquisition, with the birds remaining expressing higher working memory ratios. These results suggest that the birds omitted, particularly CN birds, had a more difficult time in learning the holeboard task throughout the acquisition phase.

While it is difficult to compare these results to other studies as, to the best of the knowledge of the experimenter, no other studies have used a similar environmental treatment, it is perhaps somewhat expected that birds exposed to a variety of different materials throughout their rearing might be best at navigating a new environment containing foreign objects and novel foodstuff. This has been demonstrated in studies where rearing in more barren environments inhibits various coping and cognitive skills (Tahamtani et al., 2015), compared to rearing in enriched environments which has numerous positive effects such as increased locomotive and spatial skills (Widowski and Torrey, 2018; Campbell et al., 2019). Furthermore, a complex environment is also more in line with the natural environment that hens have evolved from, and might therefore be most sensible in encouraging foraging and exploratory behaviours. However, in this particular study, the birds exposed to the no-choice treatment had the same amount of substrate and perches accessible to them as the choice treatment, with the exception of course being "choice". This seems to suggest that it is also the lack of choice that affects spatial cognition negatively, not only lack of resources. In other words, choice within environmental complexity may be just as important as environmental complexity itself.

Careful consideration must also be given to the treatments used within this study. While they are referred to as "choice" and no-choice", it is difficult to say for sure if the birds actually perceived their environments in this way. There is evidence in literature that birds do have preferences within their environment, particularly in regards to perches and substrate. A study by Villagrá et al. (2014) found that broiler chickens preferred sand over three other substrates and that different substrates were preferred depending on the behaviour the birds performed i.e. dust bathing, resting, etc. Another study by Nicol et al. (2009) concluded that the laying hens in their study did indeed have environmental preferences, and that these preferences could be linked to some variables used to measure welfare. Perch type has also been shown to be important to laying hens, with studies finding hen preferences for material, shape, location and height of the perch (Chen et al., 2014; Campbell et al., 2016). In the current study the perches were all placed at the same height and evenly spaced from one another. In future studies it may be useful to consider different variations of the choices given, such as placing perches at different heights and locations, or providing different colours of perches which hens have shown to have preferences for (Taylor et al., 2003).

As previously stated, the current rearing environment of this study seemed to impact the results of the birds more positively than that of the early rearing environment, which was expected to be the rearing period within a more sensitive developmental stage (Widowski and Torrey, 2018). However, a study on chickens conducted by Ericsson et al. (2016) found that while two week old and eight week old birds were most affected by an experimental stressor, eight week old birds were more likely to experience long-term effects from the stressors, such as high corticosterone responses. Therefore, if the no-choice birds from the current study experienced more stress during the early rearing environment than the choice birds, this stress may have been short-term, and thus, did not affect the birds once they were given the treatment of choice in the current rearing environment. This may also have been true for the choice birds in the early rearing environment, where choices of perches and substrate types may have been beneficial, but did not have long-lasting effects.

Another possibility as to why the current rearing environment seemed to affect the birds more than the early rearing environment may be most easily explained through how the term "early" is actually defined. A study conducted by Gunnarsson et al. (2000) found that the spatial cognition of hens was negatively affected when perch access was withheld until 8 weeks of age. This suggests that the critical period for the development of spatial cognition may lie somewhere between 0-8 weeks of age. Another study by Tahamtani et al. (2015) discovered that laying hens raised in an aviary system from 4-16 weeks displayed better spatial skills and memory in a holeboard test than birds raised in a conventional cage system from 0-16 weeks. The birds in the current study were exposed to the early rearing environment from 1-4 weeks of age, and the current rearing environment from 5-15 weeks of age. If the overlapping ages of the birds within these spatial cognitive studies are taken into consideration, one could postulate that the critical stage of spatial cognition that has the most potential for developing long-term effects occurs somewhere between the age of 5-8 weeks, seeing as the current rearing environment in this study seemed to have the most impact on the birds' success in the holeboard test. However, these studies differed quite largely from one another, making it difficult to draw any concrete conclusions. Furthermore, as this study was using novel treatments, additional studies are needed in order to say more definitively at what age the sensitive period of spatial cognition development occurs in the early life of the laying hen.

When observing the amount of birds from each treatment that passed criteria at the various periods and phases of the holeboard test, it is quite clear that the birds in the treatment CN performed the poorest. One possible explanation for this may be that the birds viewed the move from the choice treatment to the no-choice treatment as negative, due to the fact that their environment lost an aspect of complexity. A study from Bateson and Matheson (2007) found that starling birds from standard cages viewed a stimulus as more negative when compared to starlings from enriched cages. The authors also found that this difference was only observed in starlings that had received enriched cages and were then moved to standard cages, causing them to conclude that a decline in environmental quality prompted a more pessimistic bias of the birds (Bateson and Matheson, 2007). Other research has observed similar findings where birds moved from a more complex environment to a less complex environment exhibited signs of frustration that resulted in increased feather pecking and reduced plumage quality (Janczak and Riber, 2015). However these birds experienced a more drastic change through moving from a unrestricted system with attractive substrate to a restricted system with poor substrate (Janczak and Riber, 2015). Contrary to this, the birds in the present study simply lost the aspect of choice, a seemingly small change that had significant impacts. This in itself highlights how meaningful the aspect of choice may actually be for the environment of birds during rearing.

4.2. Controllability

It is well known that environmental enrichment in general provides a variety of positive effects for poultry such as improved leg health, reduced feather pecking, improved spatial cognition, reduced fear, improved immune function and reduced stress (Tahamtani et al., 2016; Brantsæter et al., 2017; Campbell et al., 2019; Pedersen et al., 2020), all of which lead to better hen welfare. Therefore, it is possible that providing the birds with an option of substrate and perch type within the choice treatments was simply another form of environmental enrichment or added complexity. However, one might not expect differences between treatments simply due to adding different types of substrates or perches if environmental complexity was the only driving factor in the results observed. Alternatively, the treatment of choice may have provided the birds controllability over their environment. Early research investigating controllability has suggested that low levels of controllability or predictability over long amounts of time could lead to chronic stress in animals, resulting in long lasting effects such as reduced vigilance and coping skills (Wiepkema and Koolhaas, 1993). A more recent study by Lucas et al. (2014) investigated two different treatment groups of rats over a prolonged period of time: rats that had control over a stressor (i.e. could escape the stressor before or while exposed to it) and rats that did not. The authors found that the rats in the controllable group were able to obtain "emotional controllability", in which they displayed reduced levels of fear, and demonstrated higher exploratory activity and a better ability to learn under stressful conditions. Conversely, rats in the uncontrollable group displayed higher levels of fear, impaired learning skills (regardless of if the task was positively or negatively reinforced), elevated HPA axis responsiveness 24 hours after being tested, and lower exploratory behaviour that was long lasting (Lucas et al., 2014). Based on these findings, it is possible that while the birds in the no-choice treatment did not necessarily have to deal with an aversive stimulus, their environment may have been perceived as more aversive than the choice environment. As a result, no-choice birds may have experienced less control over their environment than compared to birds in the choice treatment, resulting in decreased coping skills and impaired learning abilities. Furthermore, the birds from the CN treatment may reacted more strongly to the no-choice environment than NN birds, having lost an aspect of control when their environment changed from choice to no-choice and explaining their poor performance in the holeboard test. Conversely, NC birds may have gained an adaptive advantage similar to CC birds when they changed from the no-choice environment to the choice environment. Experiencing more control through the new ability to choose perch or substrate types may have then led to the higher exploratory and cognitive skills of these birds that were observed in the holeboard test. Furthermore, as controllability has been found to positively effect coping skills (Mineka and Hendersen, 1985; Wiepkema and Koolhaas, 1993; Lucas et al., 2014), choice birds may have been better at coping with the novelty of the holeboard test than no-choice birds.

4.3. Phenotypic plasticity

As the birds from the current rearing environment of choice were more successful in the holeboard test, it is possible that CC and NC birds developed higher degrees of phenotypic plasticity. That is "the extent to which an organism can change its physiology, behaviour, morphology and/or development in response to environmental cues", as described by Dufty et al. (2002). As the environment influences phenotypic plasticity, the positive aspect of choice experienced by the birds in the treatment CC and NC may have aided them in becoming more flexible in how they responded to environmental changes. According to the Predictive Adaptive Response Hypothesis, a form of developmental plasticity that is contingent on cues received during the early environment (Bateson et al., 2014), it would then be expected that the birds in CC and NC treatments that were more successful at the holeboard test would continue to experience long-term advantageous effects from the skills developed during rearing. Alternatively, NN and CN birds, which were slower to complete habituation when they were suddenly forced to habituate alone, may have displayed lower phenotypic plasticity due to the current rearing environment of no-choice.

4.4. Habituation and holeboard test

The results found during habituation to the holeboard test displayed significant differences amongst treatments groups, which was particularly interesting for the CC birds. Worms found is an important variable to assess within the habituation, given that this displays the birds' spatial awareness, exploration and foraging skills, and in many ways, their ability to adapt to a new environment and take advantage of the opportunities presented to them. This is particularly meaningful during habituation alone, when the birds cannot rely on their pen mates to learn from or to offer social support. While no differences were found within group habituation, during habituation alone, CC birds found a significantly greater number of worms than both treatment CN and treatment NN. Birds from treatment NC also showed a strong trend of finding more worms than treatment CN. These results suggest that having the current rearing environment of choice after early rearing, as both treatments CC and NC did, may have offered them some adaptable advantage in not only being able to locate food and resources in an environment with conspecifics, but also being adept at continuing to do so when these circumstances changed. The birds from the treatment CC and NC were also significantly faster at completing their habituation group sessions than birds from NN and CN, though it appeared that this was mostly a result of the test duration of the CC birds, which was the fastest of all treatment groups. At this stage in the experiment, the holeboard arena was still very new to the birds, which suggests that birds from the treatment CC were affected most positively by the novel environment and reacted least fearfully, as shown by the shortest overall test duration.

For both cup and worm latency it appeared that while CC and NC birds reached the first cup and the first worm just as quickly regardless of if they were habituated alone or in a group, NN and CN birds performed slower when habituated alone. Furthermore, CC and NC birds were significantly faster at reaching the first worm in habituation alone than NN and CN birds. These results are somewhat similar compared to worms found, as it was clearly shown that the performance of NN and CN birds was affected when their pen mates were no longer habituating with them. It also suggests that there was likely a fear aspect involved in habituating alone for the treatment groups NN and CN, as they were more cautious of the worms and cups, therefore increasing the time it took for them to begin to explore. Comparable results have been shown in a study where a line of high-anxiety related behaviour rats showed reduced stress in a modified holeboard task when tested amongst group mates (Ohl et al., 2001), similar to the NN and CN birds. Furthermore, a study by Brantsæter et al. (2016) concluded that hens reared in more complex environments displayed less fearfulness than hens raised in barren environments. Though the no-choice treatment pens in the current study were not barren, other research has shown that complexity in general reduces fearfulness in hens compared to hens with comfortable but non-complex environments (Campderrich et al., 2019). Having the current rearing environment of no-choice may have then reduced these birds' adaptability and/or increased their fear of novelty, causing them to rely more heavily on the comfort of their flock when in new environments.

In regards to the number of worms found during the acquisition, the birds in treatment CC and NC continued to find more worms than both NN and CN birds, though statistically only a trend was shown for CC finding more worms than CN. While the end trials of the acquisition phase showed that the NN bird found more worms than the CC birds, it is difficult to draw conclusions based on so few birds participating. That being said, the NN birds were still perhaps more successful in the holeboard test than the CN birds who were omitted entirely, which might suggest that the change from the choice treatment to the no-choice treatment was more detrimental to the CN birds than only receiving the treatment no-choice, as the NN birds did.

4.5. Effect on memory ratios

One aspect of this study was investigating memory ratios of the birds throughout the various phases of the holeboard test. As memory ratios are used as a means to assess spatial cognition, rearing birds with high memory ratios would be expected to increase adaptability and cognition. While no differences in memory ratios were found during the acquisition phase, total cups found and total worms found can also be considered as a means to assess learning capability. When the total cups found (including revisits) was compared between the birds remaining in the test ("in") and the birds omitted ("out") after the start, middle, or end trials, the start trials showed a trend for more cups to be found by "in" birds, while there were no significant differences between cups found during the end trials (see appendix 9.3., Table 8). Though there was a trend of differences between total cups found between the birds "in" and out" during the start trials, this is to be expected, as the holeboard test was still very new to the birds, and it is likely that personality and fear of individual birds was more of a factor during this period. However, as the end trials are important for determining the progress the birds made since the start of the holeboard test, these results are particularly interesting when also considering mean worms found, where birds "in" found significantly more worms than birds "out". These results signify that while the birds that had been omitted may not have explored quite as much as the birds remaining, they were much less successful at locating the reward cups and learning the holeboard test (see appendix 9.3., Table 9). This also suggests that even though the memory ratios did not differ significantly during the acquisition phase, the birds that remained in the test were more successful than those omitted due to better learning skills, and not necessarily because they were less fearful or more motivated to explore.

The reversal phase of the holeboard test displayed differences of reference memory between the four birds that participated, though due to the low number of birds, these results should be interpreted with caution. Of the birds remaining, the two NC birds displayed a higher memory ratio when compared to the NN bird. In other words, the NC birds were more successful at remembering which cups the worms were located during the reversal phase of the holeboard test than the NN bird. As the NC birds showed a trend of having a higher memory ratio than the CC birds, it may be possible that moving from the no-choice environment to the choice environment positively affected the NC birds, and not simply having the current rearing environment of choice. Interestingly, both NC birds and NN birds improved in their reference memory ratios from the start trials to the end trials of the acquisition, whereas CC birds performed much the same throughout. This suggests that while the different rearing environments certainly may have affected holeboard performances, other factors such as exploration, fearfulness or even the barn environment during testing, may have had an impact on the learning processes of the birds. Further tests using a similar approach but in a more controlled testing environment with more birds may help to clarify any possible treatment effects. It is also important to remember that while a higher memory ratio may signify that an animal has a higher capacity for learning that particular task, it is also the increase in memory ratio from the beginning of the test to the end of the test that is indicative of learning taking place.

As reference memory has been described as a type of long-term memory (Dudchenko, 2004; Kay et al., 2010) where an individual learns a strategy to solve a particular problem or task, the NC birds that displayed higher reference memory ratios may be more equipped to overcome obstacles through strategic learning over the course of their life. Additionally, the difference in memory ratios between individuals was observed during the reversal phase, which is often more challenging as participants must essentially forget previously learned information and re-learn a task based on the new information presented, requiring more behavioural flexibility (Dhawan et al., 2019). This suggests that when faced with this challenge or stress, the NC birds were more adequate at coping with, or more behaviourally flexible, to the change in their environment than the other birds in the reversal phase. However, the results of reference memory ratios of the treatment groups should again be interpreted cautiously, as so few birds from each treatment were tested in the reversal phase.

In terms of working memory and general working memory, no differences were seen throughout the acquisition phase or the reversal phase of the holeboard test. This may be due to the fact that poor performing birds (those that did not meet the holeboard criteria) were omitted from testing, leaving a low number of birds left to be analyzed, reducing the statistical power of the data. Omitting poor performing birds also resulted in higher performing birds being left in the holeboard test, thereby reducing the amount of variation that may have existed across treatment groups, which is also true for other variables throughout the acquisition and reversal that did not differ, such as cup latency, worm latency, and test duration. Another possibility is that time between consecutive trials of individual birds was not long enough in order for working memory to be properly analyzed. As working memory is a short-term type of memory, there must be enough time between trials in order for the memory to "reset" (Frick et al., 1995). A previous study using a holeboard test with laying hens allowed for one minute between consecutive trials for the first five trials, and one hour between consecutive trials thereafter (Nordquist et al., 2011). No differences between working memory were observed in the hens from this study, even with a one hour break between trials (Nordquist et al., 2011), though this of course could have been due to treatment. As time between consecutive trials in the current study ranged from 5-20 minutes, there was likely enough time for the birds' short-term memory to reset, though to be sure, future studies using a holeboard test may decide to provide more time between consecutive trials.

4.6. Implications

The results from this study could be implemented in commercial farm settings to promote cognitive development during rearing, which could have possible positive impacts on hen adaptability. The substrates and perches used within this study were selected particularly for their feasibility of use in a variety of production systems, as well as the fact that many of the substrates and perch types in this study are already being used in some aspect in modern day layer production. Though the results from this study indicate that the second period of early rearing (the current rearing environment) is most responsible for promoting cognitive development in laying hens, that is not to say that enrichments and environmental complexity in the early rearing period are not still important to implement in regards to other welfare aspects of the birds. Rather, results from this study suggest that while having choice environments during early rearing may have beneficial short-term effects, there may be a period slightly later in the rearing stage that is particularly important to focus on in terms of environmental complexity with the purpose of promoting potential long-term adaptability and cognitive development. Through implementing environmental complexity by way of choice in production systems during this crucial period of rear, laying hens may develop better skills to adapt and cope with challenges in their life, with the possibility of increasing spatial cognition, reducing health related problems, decreasing fearfulness of hens, and improving overall laying hen welfare (Tahamtani et al., 2015; Campbell et al., 2019; Campderrich et al., 2019). In doing so, production systems can increasingly rear birds in a way that positively benefits both the bird, as well as the producer.

4.7. Limitations

While the results from this study indicate that choice within the rearing environment does, in fact, have an affect on the spatial cognition, with possible impacts on the adaptability and fear of laying hens, certain limitations of this study should be taken into consideration. One such limitation was the choice to omit birds that were unable to meet holeboard criteria throughout the test. This was done for practical reasons, as the holeboard test itself was fairly time consuming to run, especially when birds continuously remained in holeboard trials for the maximum amount of time without finding any worms. However, if more time was available, it may have been beneficial to continue testing all birds, and instead analyze the amount of trials taken by the birds to pass each period of the holeboard test. This would allow for more variation within the data, which might illustrate any treatment effects more clearly, and could help avoid the issue of having a small number of birds in the end stages of the test that perform somewhat similarly in terms of their abilities in the holeboard test.

Similar studies assessing laying hens or birds using one form or another of the holeboard test restricted access to food for a period before testing began (Nordquist et al., 2011; Parois et al., 2017) in order to increase the likeliness that the reward given was deemed as positive and that the birds were motivated to locate them. However, during the present study it was not possible to restrict access to food for the test birds, as they were housed with other pen mates that were not participating in the test. Though this did not seem to affect the birds in the view of the experimenter, as many birds ate the mealworms readily throughout testing days, it may have resulted in a decreased motivation of some birds to find the mealworms. If this was the case, more focus from the birds may have been spent on exploring and interacting with the holeboard arena. In some cases this seemed particularly true, as certain birds would spend the majority of their time pecking at the arena walls and floor, even once they had successfully associated the cups in the arena with containing mealworms. However, this type of pecking behaviour could also be a result of stress, which can cause birds to become easily distracted with other aspects of their environment (Rogers, 2010). Furthermore, other examples of holeboard tests have not restricted food prior to testing, and this did not appear to affect end results (Tahamtani et al., 2015; Hewlett and Nordquist, 2019).

While not a limitation *per se* it should also be pointed out that the treatments and phases of rearing for this type of test could be designed in any number of ways. Specifically, and perhaps most importantly in regards to narrowing down the period of rearing that is most susceptible to cognitive development, only two distinct rearing periods were used within this study. If questions regarding critical developmental phases in rearing are to be answered, future studies may benefit from using the treatments of choice and no-choice over three or four periods within rearing. Furthermore, as choice seems to have a positive impact on birds during rearing, it may also be beneficial to consider what else in the environment can represent choice for the birds. It may be possible to provide several different foodstuffs, or offer different lighting intensities throughout the pen. However, while continuing to explore the aspect of choice in the rearing of birds, it is important to keep in mind the feasibility of implementing such choices within a production system.

5. Conclusion

The results from this study demonstrate that through rearing laying hens using environmental complexity in the form of choice, cognitive development, particularly in regards to spatial cognition, can be better supported. This in turn may result in birds with greater adaptive abilities that are needed to navigate life in production systems. As the current study found that birds reared with choice during 5-15 weeks of age displayed better performance in a holeboard test, particular attention to cognitive development during this time may be warranted. Through supplying choice during this stage of life, laying hens may be more equipped to deal with new environments and experiences due to improved coping skills and less fearfulness in novel situations. Furthermore, while providing birds with environmental complexity throughout life is important, this study has also shown that increasing environmental complexity over the course of rearing may have additional benefits. The early rearing environment in the current study did not seem most effective at rearing hens for success in a holeboard test, though positive short-term effects may still have been achieved. Further studies are needed to determine when exactly the critical stage of learning and cognitive development takes place in the modern day laying hen, and if implementing choice during rearing can result in long-term effects on adaptability.

6. Popular scientific summary

Chicken and egg production is a vital part of the global agriculture system, with more than 23 billion chicken in the world. With so many of these animals in production systems, it is important that they are raised in a way that supports their development, health and well-being. Laying hens are faced with many challenges throughout their lives where proper coping skills and adaptability are needed. Through raising laying hens in environments that support the development of these skills, we can better prepare them to face these challenges. This study used a holeboard test to investigate whether giving hens different choices of litter and perches in their home pens better supported their learning and memory development compared to hens that were not given choices of substrate and perches. The holeboard test involved an arena with nine cups, where three of these cups contained worms, and the rest remained empty. The birds were then taught to locate these three cups. During the second part of the test, three different cups contained worms, and the birds had to learn to instead find these cups. The results from the study found that birds that received different choices of litter and perches from the ages of 5-15 weeks performed better in the holeboard test, which may suggest that they are more skilled at adapting to new environments and experiences. In the future, studies should investigate if the positive effects of giving different choices of litter and perches are long lasting for the laying hen.

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9. Appendix

9.1. Appendix 1

Summary of test variable means

Table 5. Summary of means and standard error of means (±SEM) for variables of treatment groups (CC, NC, CN, NN) within habituation group and alone sessions and holeboard test phases, which include trials 1-4 of the acquisition (start trials), trials 11-21 of the acquisition (end trials), and the reversal. Cup latency, worm latency and test duration are represented in seconds, worms are represented in number of worms, and memory means are represented as ratios. Birds from treatment CN were not successful at completing the acquisition and thus, could not be analyzed further (NA).

	CC	NC	CN	NN
	Mean ± SEM	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
Cup latency				
Habituation Group	34.82 ± 13.07	29.32 ± 11.01	34.81 ± 13.07	32.04 ± 12.03
Habituation Alone	38.54 ± 18.16	23.24 ± 10.85	60.57 ± 28.54	58.77 ± 27.69
Acquisition Start trials	13.30 ± 7.27	9.39 ± 4.86	26.68 ± 25.51	4.58 ± 3.15
Acquisition End trials	3.88 ± 2.46	10.56 ± 7.84	NA	2.67 ± 2.93
Reversal	2.13 ± 1.53	6.33 ± 3.22	NA	6.02 ± 4.33
Worm latency				
Habituation Group	59. 40 ± 20.13	43.17 ± 14.63	51.52 ± 17.46	43.45 ± 14.73
Habituation Alone	46.50 ± 18.36	30.88 ± 12.19	117.84 ± 46.54	84.48 ± 33.36
Acquisition Start trials	48.59 ± 19.78	49.14 ± 18.75	129.18 ± 90.46	28.81 ± 14.51
Acquisition End trials	16.07 ± 7.15	27.52 ± 12.64	NA	20.09 ± 15.49
Reversal	6.21 ± 3.32	16.64 ± 6.29	NA	14.81 ± 7.92
Test duration				
Habituation Group	262.73 ± 21.25	291.67 ± 8.33	300.00 ± 0.00	300.00 ± 0.00
Habituation Alone	278.29 ± 16.89	282.08 ± 14.06	296.92 ± 3.08	296.46 ± 3.54
Acquisition Start trials	256.61 ± 14.99	247.16 ± 15.33	289.38 ± 10.63	113.27 ± 28.32
Acquisition End trials	122.89 ± 60.77	158.42 ± 77.48	NA	152.42± 129.92
Reversal	88.24 ± 19.23	130.88 ± 20.17	NA	125.00 ± 27.24
Worms				
Habituation Group	1.30 ± 0.33	1.53 ± 0.38	0.89 ± 0.23	1.12 ± 0.29
Habituation Alone	2.92 ± 0.72	2.03 ± 0.52	0.77 ± 0.24	1.04 ± 0.30
Acquisition Start trials	1.82 ± 0.18	1.78 ± 0.20	0.63 ± 0.26	1.81 ± 0.26
Acquisition End trials	2.09 ± 0.17	2.52 ± 0.08	NA	2.64 ± 0.15
Reversal	3.0 ± 0.00	2.80 ± 0.16	NA	2.80 ± 0.20
Working Memory				
Acquisition Start trials	0.64 ± 0.22	0.55 ± 0.18	0.29 ± 0.25	0.59 ± 0.25
Acquisition End trials	0.75 ± 0.16	0.69 ± 0.18	NA	0.65 ± 0.26
Reversal	0.68 ± 0.07	0.71 ± 0.06	NA	0.77 ± 0.09
General Working Memory				
Acquisition Start trials	0.68 ± 0.11	0.71 ± 0.10	0.55 ± 0.17	0.72 ± 0.14
Acquisition End trials	0.71 ± 0.15	0.74 ± 0.14	NA	0.72 ± 0.27
Reversal	0.65 ± 0.05	0.76 ± 0.03	NA	0.76 ± 0.10
Reference Memory				
Acquisition Start trials	0.41 ± 0.08	0.38 ± 0.08	0.25 ± 0.13	0.35 ± 0.10
Acquisition End trials	0.39 ± 0.06	0.46 ± 0.07	NA	0.45 ± 0.11
Reversal	0.35 ± 0.02	0.42 ± 0.02	NA	0.35 ± 0.02

9.2. Appendix 2

Working memory means of birds from each treatment remaining in test and birds omitted

Table 6. Summary of means and standard error of means (±SEM) of the working memory ratios of birds remaining in ("in") the holeboard test and birds omitted from the test ("out") after each of the three phases of acquisition i.e. start trials, middle trials, and end trials, from each **treatment group**.

	Working memory							
	CC in	**CC out**	**NC in**	**NC out**	**CN in**	**CN out**	**NN in**	**NN out**
Start trials	0.64 ± 0.06	N/A	0.63 ± 0.06	0 ± 0	N/A	0.29 ± 0.13	0.74 ± 0.08	0.17 ± 0.17
Middle trials	0.75 ± 0.08	0.34 ± 0.07	0.74 ± 0.05	0.29 ± 0.08	N/A	N/A	0.78 ± 0.07	0.55 ± 0.10
End trials	0.92 ± 0.06	0.67 ± 0.07	0.75 ± 0.05	0.63 ± 0.05	N/A	N/A	0.65 ± 0.09	N/A

9.3. Appendix 3

Working memory means, total cups found (including revisits) and worms found of all birds remaining in test and birds omitted

Table 7. Summary of means, standard error of means (±SEM) and test statistics of the working memory ratios of **all** birds remaining in ("in") the holeboard test and birds omitted from the test ("out") after each of the three phases of acquisition i.e. start trials, middle trials, and end trials. Number of birds in start, middle and end trials are as follows: 21, 17, 8 (in); 27, 31, 40 (out).

	Working memory		
	In	**Out**	**Test statistics**
Start trials	0.66 ± 0.07	0.19 ± 0.11	$F_{1, 19}$= 20.57, P= 0.0002
Middle trials	0.75 ± 0.06	0.37 ± 0.06	$F_{1, 15}$= 23.57, P= 0.0002
End trials	0.77 ± 0.06	0.65 ± 0.06	$F_{1, 6}$= 2.53, P= 0.16

Table 8. Summary of means, standard error of means (±SEM) and test statistics of the total number of cups visited (including revisits) per trial of **all** birds remaining in ("in") the holeboard test and birds omitted from the test ("out") after each of the three phases of acquisition i.e. start trials, middle trials, and end trials. Number of birds in start, middle and end trials are as follows: 21, 17, 8 (in); 27, 31, 40 (out).

Total number of cups visited (including revisits)			
	In	**Out**	**Test statistics**
Start trials	9.54 ± 0.65	6.56 ± 1.33	$F_{1, 19}$= 4.08, P= 0.06
Middle trials	8.44 ± 0.68	6.50 ± 0.64	$F_{1, 15}$= 4.57, P= 0.05
End trials	9.75 ± 0.87	7.80 ± 0.87	$F_{1, 6}$= 2.50, P= 0.17

Table 9. Summary of means, standard error of means (±SEM) and test statistics of the number of worms found per trial of **all** birds remaining in ("in") the holeboard test and birds omitted from the test ("out") after each of the three phases of acquisition i.e. start trials, middle trials, and end trials. Number of birds in start, middle and end trials are as follows: 21, 17, 8 (in); 27, 31, 40 (out).

Worms found			
	In	**Out**	**Test statistics**
Start trials	1.99 ± 0.20	0.44 ± 0.30	$F_{1, 19}$= 32.00, P<0.001
Middle trials	2.35 ± 0.19	0.98 ± 0.18	$F_{1, 15}$= 28.03, P< 0.001
End trials	2.84 ± 0.18	1.91 ± 0.18	$F_{1, 6}$= 14.21, P= 0.009

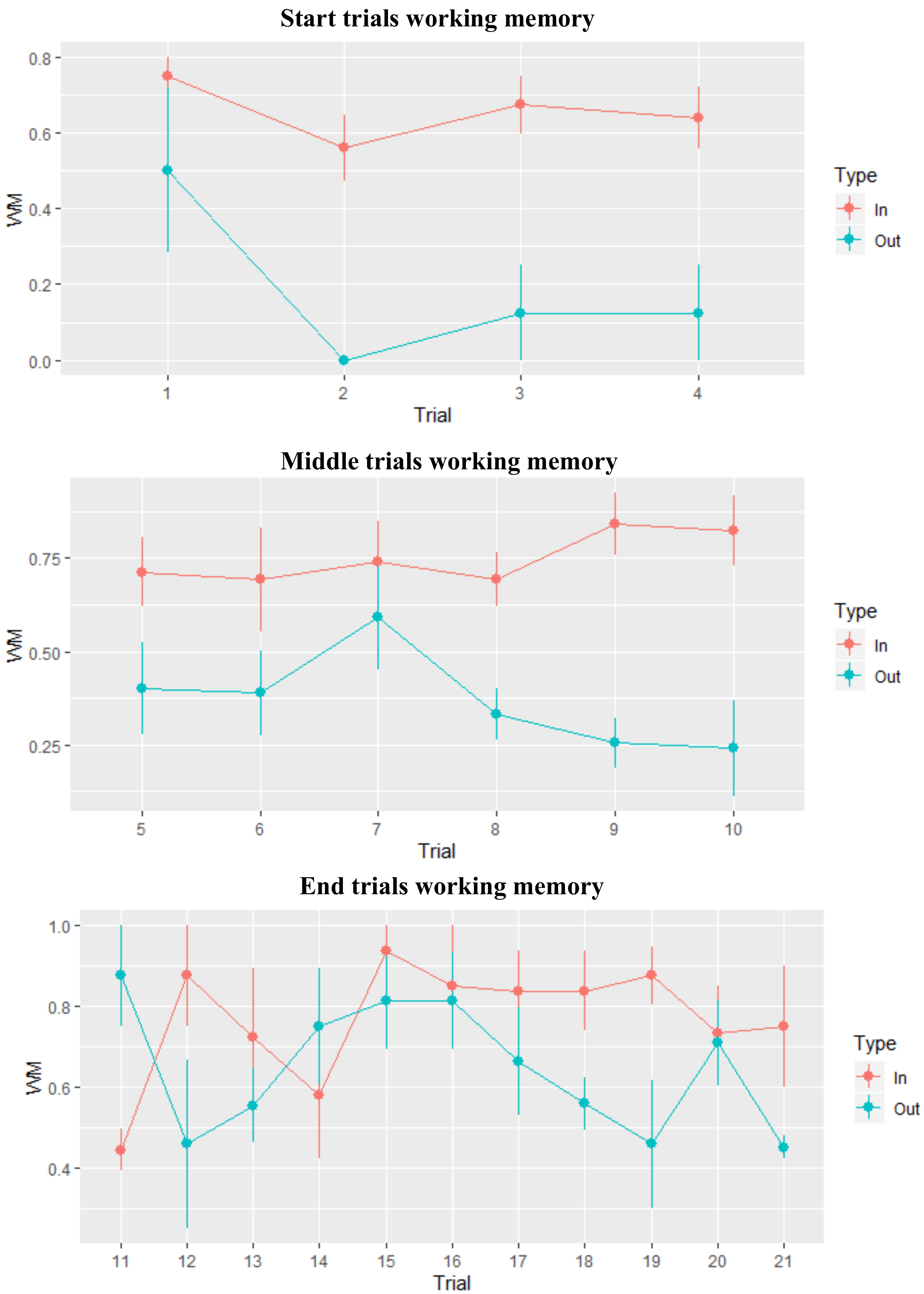

Fig. 12. Mean working memory ratios (WM) for birds that remained in the holeboard test ("in") and birds that were omitted from further testing ("out") in each phase of the acquisition (start trials, middle trials, end trials).