Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2013:56

Effects of environmental complexity during rearing and laying on fearfulness, spatial cognition and neural plasticity in laying hens

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Abbreviations and definitions

ACTH	Adrenocorticotropic hormone
ADP	Adaptive developmental plasticity
aE	Aviary-reared hens housed in enriched furnished cages
AHN	Adult hippocampal neurogenesis/Plasticity
aS	Aviary-reared hens housed in standard furnished cages
AVT	Vasotocin
cE	Cage-reared hens housed in enriched furnished cages
CRH	Corticotropin-releasing hormone
cS	Cage-reared hens housed in standard furnished cages
DCX	Doublecortin
EU	European Union
GR	Glucocorticoid receptors
HF	Hippocampal formation
H:L	Heterophil:lymphocyte
HPA	Hypothalamic-pituitary-adrenal
IHC	Immunohistochemistry
L(M)M	Linear (mixed effects) models
MR	Mineralocorticoid receptors
PAR	Predictive adaptive response
PBS	Phosphate-buffered saline
PVN	Paraventricular nucleus

List of papers

Paper I

Lucille Dumontier, Andrew M. Janczak, Tom V. Smulders, Randi O. Moe, Judit Vas, Janicke Nordgreen (2022).
Early life environment and adult enrichment: Effects on fearfulness in laying hens.
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Paper II

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Effects of the rearing environment complexity on laying hens' spatial cognition: A holeboard test approach.
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Paper III

Lucille Dumontier, Andrew M. Janczak, Tom V. Smulders, Rebecca-Leigh Railton, Janicke Nordgreen
Environmental complexity and fearfulness are associated with spatial cognition and hippocampal plasticity in laying hens.
Manuscript

Paper IV

Janicke Nordgreen, **Lucille Dumontier**, Tom V. Smulders, Judit Vas, Rupert Palme, Andrew M. Janczak.
Effects of rearing and of the adult environment on HPA-axis responsivity and feather score in laying hens.
Submitted to Applied Animal Behaviour Science.

Abstract

Due to an increased demand from citizens, the egg industry in the European Union is moving towards more welfare friendly housing systems. However, these environments are often more complex, and hens are still exposed to a great variety of stressors, potentially resulting in the experience of chronic stress. Environmental complexity and enrichment in young animals are known to promote cognitive abilities and better stress resilience. However, effects in the long term and at different stages of life have been very little described in laying hens. The aim of this project was to investigate how environmental complexity during rearing (cage or aviary) and the production period (standard or enriched furnished cages) would affect laying hens' characteristics. The thesis particularly focuses on effects on fearfulness, stress responsivity, spatial cognition, and hippocampal plasticity. Overall, the results show that both the rearing and the adult environment affected laying hens when measured halfway through and at the end of the production period. Higher environmental complexity during rearing primarily improved the hens' spatial cognition, while the provision of enrichment during the production period decreased fearfulness and levels of corticosterone in plasma. Hippocampal plasticity was affected by both rearing and production environments, with effects varying between the two subregions of the hippocampal formation (HF). Survival of cells expressing doublecortin was enhanced in the caudal HF for aviary-reared birds housed in enriched cages, and in the rostral HF for cage-reared hens housed in enriched cages. The cell density in the caudal HF was positively correlated with the distance walked in an open field arena, and birds performing the detour had a higher cell density in the caudal HF than birds not making the detour. Put together, these results suggest that increased environmental complexity both during rearing and the production period can positively affect laying hens, with rearing effects on cognitive abilities being long-lasting.

Norsk sammendrag

På grunn av økt etterspørsel fra innbyggerne beveger eggindustrien i Den europeiske union seg mot mer velferdsvennlige systemer for hold av verpehøner. Disse miljøene er ofte mer komplekse, og hønene kan fremdeles bli utsatt for en stor variasjon av stressorer som kan føre til kronisk stress. Miljømessig kompleksitet og berikelse hos unge dyr er kjent for å fremme kognitive evner og bedre stressmotstand, men effektene på lang sikt og på ulike livsstadier har i liten grad blitt beskrevet hos verpehøner. Målet med dette prosjektet var å undersøke hvordan miljømessig kompleksitet i oppalsperioden (bur eller aviarier) og produksjonsperioden (standard eller berikede innredede bur) ville påvirke verpehøners egenskaper. Arbeidet fokuserer spesielt på effekter på fryktsomhet, stress-responsivitet, romlig læring og plastisitet i hippocampus. Samlet sett viser resultatene at både oppalsmiljøet og voksenmiljøet påvirket verpehøner når effektene ble målt halvveis og ved slutten av produksjonsperioden. Høyere miljømessig kompleksitet under oppdrett forbedret hovedsakelig hønenes romlige læring, mens tilførsel av berikelse i produksjonsperioden reduserte fryktsomhet og nivåer av stresshormonet kortikosteron i plasma. Plastisitet i hippocampus ble påvirket av både oppals- og produksjonsmiljøene, med varierende effekter i rostrale og kaudale del av hippocampus. Overlevelsen av celler som uttrykker plastisitetsmarkøren doublecortin ble forbedret i den kaudale delen av hippocampus for aviar-oppalede fugler som bodde i berikede bur, og i den rostrale delen av hippocampus for bur-oppalede høner som bodde i berikede bur. I tillegg var det en positiv korrelasjon mellom hvor langt fuglene gikk i en åpen arena og celletettheten i kaudale hippocampus. Fugler som klarte en detour test hadde også høyere celletetthet i dette hjerneområdet enn fugler som ikke klarte testen. Disse resultatene indikerer at økt miljømessig kompleksitet både under oppal og produksjonsperioden kan ha en positiv effekt på verpehøner, der effekter fra oppal på kognitive evner også sees hos voksne individer.

1 Introduction

The domestic chicken (*Gallus gallus domesticus*) was domesticated from the red jungle fowl (*Gallus gallus*) at least 3,200 years ago (Peters et al., 2022). It has since become the most produced livestock in the world, with 25.9 billion chickens kept for meat and egg production in 2021 (FAO, 2023). With the human population growing the pressure on food production is increased. To supply the increasing demand for food, genetic lines of chickens have been selected for specific production traits, such as rapid growth or high egg production, with little considerations about side effects on animal welfare and behaviour (Gunnarsson, 2018). As a result, chickens have been kept under highly intensive production conditions for decades. Though barren housing is still in use in some parts of the world, a growing proportion of countries are moving towards more welfare-friendly housing systems. Conventional cages for laying hens, also known as battery cages, have been banned in the European Union (EU) since 2012 (Council of the European Union, 1999), and the "End the Cage Age" initiative is now influencing legislation to move towards cage-free systems for a majority of production animals, including chickens (Compassion in World Farming International, 2022). The alternatives to conventional cages offer the benefits of more stimulation, potentially resulting in better stress resilience, reduced fearfulness and improved cognitive abilities, but these systems are also more complex for the birds and experiences during the rearing phase will be important to prepare the birds for their housing during the production period. The environment experienced during early-life being a major determinant in the development of an individual, a good understanding of the effects of the rearing environment on the hens' characteristics and subsequent life is crucial to improve their welfare. There is however little basic knowledge on how the environmental complexity experienced by the hens during rearing could affect their characteristics on the long-term, especially regarding cognitive abilities and stress responsivity. In this thesis, we investigated the medium- and long-term effects of environmental complexity during rearing and adulthood on laying hens' spatial cognition, fearfulness and stress.

1.1 Egg industry

1.1.1 General overview

Worldwide, about 8.1 billion laying hens were kept for egg production in 2021, with China being the main egg producer with over 3 billion hens (FAO, 2023). Norway housed over 4.6 million laying hens in 2022, which represent an increase of 45% over the past 20 years (SSB, 2023). This increase in production mirrors the rise in egg consumption, and husbandries should provide an adequate environment for the hens at every life-stage. In the egg industry, eggs are usually incubated in hatcheries until hatching of the chicks. Chicks are then processed and vaccinated, and female day-old chicks are transferred to rearing facilities. The rearing period lasts until the pullets reach sexual maturity at about 15-18 weeks of age, and young laying hens are then transported to the laying farms, right before the onset of lay. The hens will remain at the laying farm for the rest of the production period, which ends when the hens reach 70-80 weeks of age depending on the breed. As of 2021, most hens spent their entire life in conventional or furnished cage systems (Schuck-Paim et al., 2021b). In the EU, almost half of the hens are still kept in furnished cages (44.9%), the other half being distributed between barns (35.6%) and free-range or organic systems (19.4%) (European Commission, 2023). In Norway, the majority of the hens are now housed in cage-free systems, with only 6 % of the hens housed in furnished cages (Animalia, 2022). I will describe in the following subsections the characteristics of the different systems used to rear chicks and house laying hens. Unless specified otherwise, I will focus only on systems found in the EU.

1.1.2 Housing systems during rearing

There are no specific regulations for chick rearing in the EU, which means the chicks fall under the general directive on the protection of farm animals (Council of the European Union, 1998). This directive covers all farm animals, which means it does not provide species specific requirements in terms of housing for chicks. This leads to massive discrepancies in the characteristics of the different systems, but rearing systems can be split into two main categories: the cage systems and the non-cage systems. The latter are also referred to as alternative or loose systems.

1.1.2.1 Cage systems

A typical cage system usually consists of small, enclosed spaces with a wire mesh floor, often stacked in tiers. Manure belts between the tiers allow for the collection of droppings and feathers, and nipple drinkers provide access to water inside the cage. Feed is provided via a feed trough running along the front of the cages. Stocking density is not regulated by the law, but it normally ranges between 30 and 37 birds/m^2 (EFSA Panel on Animal Health and Animal Welfare, 2023). Some systems provide the birds with additional features, such as perches, pecking stones or chick paper. Chick paper is generally lined on the floor of the cages before chicks' arrival and allow the accumulation of droppings and feed, providing a substrate for foraging and dustbathing, as well as ensuring the effect of the coccidiosis vaccine. Perches allow the birds to perch, which is a highly motivated behaviour and contributes to the development of the chicks' three-dimensional use of the environment (see section 1.3). However, these are not legal requirements, and most cage systems used to rear chicks are still barren.

1.1.2.2 Non-cage systems

There are several alternative systems to cages. These alternative systems can be divided in different categories based on their degree of environmental complexity as follows: floor systems, single tier systems and multi-tier or aviary systems. In a floor system, chicks are housed in a barn with all resources (feed, water) distributed on the same level, i.e. the floor of the house. Feed and water lines heights can be adjusted according to the growth of the chicks to guarantee easy access to resources at any stage of the rearing period. Single-tier systems are similar to floor systems but are furnished with additional structures like perches and elevated platforms. The height of these structures can be adjusted, and ramps can be used to facilitate the access of the chicks to the structures above floor level (Norman et al., 2021). Chicks are generally restricted to a section of the barn during the brooding period and gradually given access to more space as they grow.

In multi-tier systems, chicks are usually confined to the first or second tiers for the first few weeks. This is to ensure the chicks are big enough to move between the tiers and access the resources before being released into the house. Indeed, resources are typically distributed between the different tiers of the aviary, with the litter area on the floor of the house, the food and water in the middle tiers, and the roosting area on the upper tier. This set-up appeals to the natural behaviour of the chicks, which seek

height to rest at night. Once released from the first and second tiers, the chicks can move freely within and between the different tiers of the system, which enhances the development of their physical and cognitive abilities (see section 1.3). Tiers are usually furnished with feed and water lines, and perches are distributed between the tiers.

For all systems, the floor of the rearing house is usually covered with a substrate, such as wood shavings. At arrival, stocking density can be over 100 birds/m^2 and then decrease to 13-32 birds/m^2 by the end of the rearing period (EFSA Panel on Animal Health and Animal Welfare, 2023).

1.1.3 Housing systems during laying

Housing systems for the laying phase are fairly similar to those used for the rearing phase, with some adjustments to allow for egg laying and collection. However, unlike for the chicks, the legislation in the EU has stricter criteria for laying hens' housing and stipulates specific requirements in term of environmental characteristics. Following evidence from research that hens were restricted in their behaviours, conventional cages have been banned in the EU and the UK since 2012. Other non-European countries such as New Zealand, Australia and some of the states in the United States are now in the process of banning conventional cages as well. As of today, furnished cages are still an alternative to conventional cages and are still in use in Europe. There is however a strong pressure to move towards alternative housing systems, such as barn and free-range, and cages are being phased out.

In enriched cage systems, the Council Directive 1999/74/EC stipulates that hens must have access to perches, litter material, a claw-shortening device and a nest box in addition to feed and water (Council of the European Union, 1999). Each hen must have access to an area of at least 750 cm^2, with a minimum of 15 cm of perch and 12 cm of feed trough. The floor of the cage is usually tilted, so the eggs can roll to the front of the cage and be collected by an egg belt.

For all alternative systems, hens must be housed with a maximum stocking density of 9 hens/m^2 and at least 1/3 of the floor of the house must be covered with litter (e.g., straw, wood shavings). In free-range and organic systems, outdoor access must be provided. The rules are slightly stricter for organic production, with a maximum

stocking density of 6 hens/m^2 and 18 cm of perch per hen (European Commission, 2008).

1.2 Welfare in laying hens

As seen in the previous sections, the environment experienced by chicks and hens can be very different from one farm to another as there is a great diversity of housing systems. The industry and housing systems were shaped in the first place to maximise productivity and were very intensive, but the public concerns about the welfare of the birds are now driving some changes.

Interest in farm animal welfare has been growing since the 60s, notably with Ruth Harrison's book "Animal Machines" (1964). This book brought to light the practices used in animal production and became a starting point for measures to protect animals' integrity. Following propositions made by the Brambell Committee (1965), the Farm Animal Welfare Council (1979) advised to revise the Welfare Codes by providing farm animals with "*freedom from hunger, thirst or malnutrition; appropriate comfort and shelter; prevention, or rapid diagnosis and treatment, of injury and disease; freedom to display most normal patterns of behaviour; and freedom from fear*". This early version of the Five Freedoms has later been revised to the one currently recognized worldwide:

1. Freedom from hunger, thirst, and malnutrition, by ready access to water and a diet to maintain health and vigour.
2. Freedom from physical and thermal discomfort, by providing an appropriate environment.
3. Freedom from pain, injury, and disease, by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour, by providing sufficient space, proper facilities and appropriate company of the animal's own kind.
5. Freedom from fear and distress, by ensuring conditions and treatment, which avoid mental suffering.

These freedoms were meant to offer a framework to protect farm animals from abuse and significant improvements have been made in terms of animal welfare since they were first published. Animal welfare is a complex notion, and several attempts have been made to define it. Broom (1986) defined an individual's welfare as "*its state as regards its attempts to cope with its environment*". More recent definitions go even

further and consider not only the coping of the individual with its environment, but its positive experience. This is for example the case of the definition from the Anses (2018) which defines the welfare of an animal as "*its positive mental and physical state as related to the fulfilment of its physiological and behavioural needs in addition to its expectations. This state can vary depending on the animal's perception of a given situation.*". This is in line with other views on animals' quality of life, such as "a life worth living" (Farm Animal Welfare Council, 2009). By taking into account the mental state of the animal, these definitions also stress the importance of the individual's subjective experience in animal welfare.

1.2.1 Welfare problems in laying hens

Despite recent improvements, life in an intensive environment still gives rise to welfare issues in laying hens. Although chickens were domesticated 3,200 years ago, their behavioural repertoire is still very similar to the one of the red jungle fowl (Collias & Collias, 1967). The environment experienced by the hens is however very different from the one of their forest-dwelling ancestors (Collias & Collias, 1967) and each housing system presents its own challenges.

One of the main challenges to hens' welfare is behavioural restrictions. This is particularly the case in cage systems. Though cage systems present advantages in terms of production costs and allow for housing hens in small social groups, which is closer to the social structure of the red jungle fowl, the opportunity to perform all behaviours from their repertoire is quite low (Lay et al., 2011). In the battery cages previously used in the EU, laying hens had access to only 450 cm2/hen which restricted them a lot in the variety of behaviours they could express (see Figure 1). This led to the move towards furnished cages to provide them with more space, but the 750 cm^2 the hens have access to are still not enough to ensure that they can freely move, preen, or flap their wings. These behavioural restrictions impair their welfare and lead to stress and frustration (Appleby & Hughes, 1991; Schuck-Paim et al., 2021a). In addition, despite the provision of a litter area in furnished cages, dustbathing is still a challenge as the platform is not accessible all day in some cage types to avoid egg laying on the platform. Dustbathing material is also quickly kicked off the dustbathing platform or mat after being provided, limiting the opportunity to dustbathe. With regards to behavioural expression, alternative (i.e., cage-free) housing systems are more welfare friendly. Hens have access to at least 1,100 cm^2 per

hen and can fulfil more of their physical needs by stretching, dustbathing, and perching.

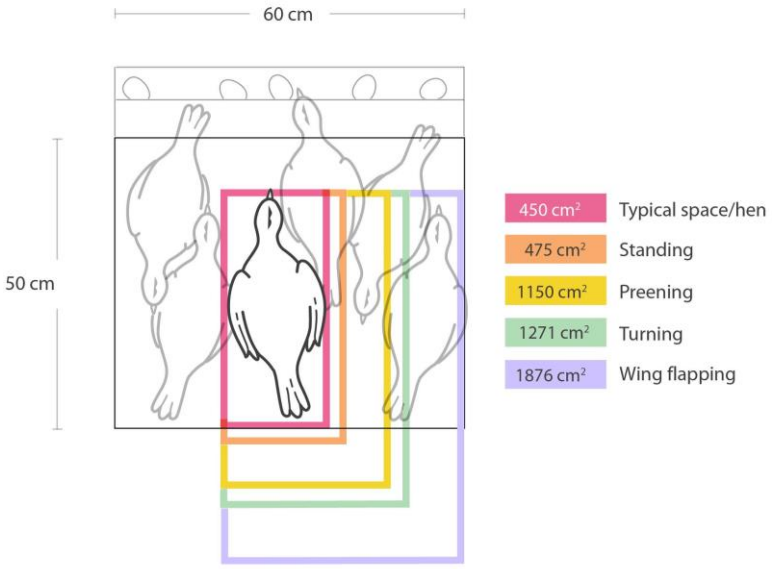

Figure 1: Area required to perform several comfort behaviours relative to the space typically available in conventional cage systems. Schematic representation from Schuck-Paim et al. (2021a), adapted from Dawkins and Hardie (1989).

Some of the other main challenges to welfare in alternative housing systems are severe feather pecking and keel bone fractures (EFSA Panel on Animal Health and Animal Welfare, 2023). The occurrence of keel bone damage is higher in aviaries due to an increased risk of collision with the structure while moving between the different tiers (Stratmann et al., 2015). But collision is not the only factor causing keel bone fracture as it is also found in cage systems and can be due to, among other things, elevated egg production (Toscano et al., 2020). Regardless of the cause, keel bone fractures are known to induce pain and chronic stress in layers (Armstrong et al., 2020). The occurrence of feather pecking is influenced by environmental (litter provision, light intensity, enrichment) and genetic factors, and feather pecking can lead to damage to the skin and ultimately to cannibalism (Savory, 1995). As a way of reducing the consequences of feather pecking, some countries practice beak trimming, which consists of removing the tip of the beak by using a hot blade or infrared treatment. Beak trimming is a welfare issue as the beak is sensitive and trimming can induce acute and chronic pain to the hens, in particular when the hot

blade method is used (Gilani et al., 2013; Marchant-Forde et al., 2008). When it comes to traits measured on individual birds, fearfulness has been found to be linked to the likelihood of feather pecking (de Haas et al., 2014a; Guinebretière et al., 2020; Kjaer & Vestergaard, 1999).

Fearfulness can be defined as the individual's predisposition to be easily frightened (Boissy, 1995; Jones, 1996), with fearful individuals displaying fear responses which are stronger and longer than less fearful individuals. Though fearfulness is adaptive to protect the animal from danger by avoiding potential threats, it can be a welfare challenge if the response to the stimuli is not appropriate (Boissy, 1995; Jones, 1996; Mills & Faure, 1990). In addition to increasing the risk of feather pecking within the flock, high levels of fear can lead to panic, with an increased risk of collision and smothering (Barrett et al., 2014; Bright & Johnson, 2011). Birds can also scratch each other, ending in open wounds and potentially cannibalism. These consequences lead to a higher mortality rate and can induce pain to the birds. Increased fearfulness is also associated with negative emotional states (Boissy, 1995) and can result in a higher risk of keel bone fracture (Harlander-Matauschek et al., 2015).

1.2.2 Benefits of environmental enrichment

One way to improve the welfare of laying hens is to provide environmental enrichment. In this thesis, environmental enrichment is defined as a modification of the environment of the animals which results in an improvement of their biological functioning (adapted from (Newberry, 1995)). Enriched environments can therefore be considered as more complex than barren environments, as they usually present additional objects or structures. Battery cages were banned in the EU to the profit of furnished cages as barren environments were shown to lead to a variety of welfare problems, especially due to the lack of opportunities to express natural behaviours (Widowski et al., 2016). Compared to barren conventional cages, the provision of perches, dustbathing areas and nests in furnished cages represent a source of enrichment since they allow for the expression of more behaviours. In the same way, alternative housing systems are more enriched and complex than cage systems as they allow the hens to express a larger panel of behaviours, such as exploration and foraging (Lay et al., 2011; Rodenburg et al., 2008). In addition to positive effects on the range of behaviours that can be expressed, environmental enrichment is also known to affect fearfulness, feather pecking and stress responsivity. For example, the provision of litter and foraging substrate in the environment decreased the

occurrences of feather pecking both in adult laying hens (Aerni et al., 2000) and pullets (de Haas et al., 2014b; Huber-Eicher & Wechsler, 1997). Exposure to environmental enrichment by providing access to perches, an outdoor range or different types of litter has been shown to be an efficient way of reducing fearfulness (Jones, 1992; Kjaer & Vestergaard, 1999; Nazar & Marin, 2011). Similarly, enriching the environment by providing various objects in the pen also contributed to decreasing fearfulness in young chicks (Jones, 1982). Providing access to elevated structures, perches, litters and foraging material decreased startle responses and the stress responsivity of laying hens (Ross et al., 2020). Associations between enrichment and cognition have also been found in several farm animal species. In pigs, housing in an enriched environment improved working memory in a holeboard task (Bolhuis et al., 2013). Likewise, enriching the environment of chicks by providing them with outdoor access increased their learning speed in a Y-maze task and promoted higher levels of exploration (Tobias Krause et al., 2006). Providing access to a 3-dimensional environment during rearing affected the depth perception of chicks up to 16 weeks of age (Jones et al., 2023). All of this suggest that increasing environmental complexity or providing environmental enrichment can be an efficient way to improve the life of laying hens. The effects of enrichment and environmental complexity could also be long-lasting if provided during the rearing period.

1.3 Developmental plasticity and rearing environment

During early stages of life, the individuals are very sensitive to their environment. Because of this plasticity, events experienced during the early life can affect the behavioural and physiological development of the individuals. Studies showed that exposure to hormones (e.g. corticosterone) or to light during incubation could affect the birds' development, like feather pecking or filial imprinting (Nordgreen et al., 2006; Riedstra & Groothuis, 2004). Filial imprinting is a well-studied phenomenon during which chicks develop a strong preference for a conspecific or object (Bateson, 1966; Bolhuis, 1991). Imprinting takes place during a sensitive time window, within 48 h post hatching in the domestic chicken (Bolhuis, 1991). Chicks develop a durable affinity which will influence their development, such as mate choice and social and feeding behaviour (Edgar et al., 2016). The phenotype of an individual is thus the result of its genetics, but also of its various experiences (Rodenburg, 2014). Understanding which traits can be affected by the early-life environment and how long the effects can last is therefore interesting. From a more applied perspective, getting a good understanding of how the rearing environment affects the

development of hens is important to produce birds that are more stress resilient and less likely to develop problem behaviours. For instance, the development of feather pecking seems to be strongly affected by the provision of litter and foraging material during the rearing period, with birds reared without showing higher levels of feather pecking and plumage damage during the laying period (de Haas et al., 2014b; Huber-Eicher & Wechsler, 1997; Tahamtani et al., 2016). Rearing chicks with dark brooders also appears to decrease the incidence of feather pecking compared to rearing with regular brooders (Gilani et al., 2012; Riber & Guzman, 2017).

As described in section 1.1.2, rearing environments for chicks can be diverse and have very distinct characteristics. These differences in characteristics lead to housing systems which present different degrees of complexity, the cage-systems being much less complex than alternative systems. The complexity of the environment is known to affect the development of several traits of the individual, such as fearfulness, cognitive abilities, and neural plasticity. Previous studies showed that hens reared in an aviary had lower levels of fear than cage-reared hens in a novel object test at 19, 21 and 23 weeks of age (Brantsæter et al., 2016a; Brantsæter et al., 2016b). The addition of environmental enrichment has also been found to reduce stress is several species (Carlstead & Shepherdson, 2000; Young, 2003), and individuals reared in a barren environment may therefore experience a higher stress level than those housed with enrichment. By increasing competition for resources and not enabling the expression of highly motivated behaviours, the lack of enrichment during the rearing phase could also act as a stressor. This potentially could have consequences for the functioning of the individuals' hypothalamic-pituitary-adrenal (HPA) axis later in life. More details on the HPA-axis and stress physiology can be found in section 1.5.

The complexity of the environment can also affect the development of the birds' cognitive abilities. This is particularly important for hens housed in alternative systems, as they need to navigate and find resources in a relatively complex environment (Lay et al., 2011). Access to elevated structures or perches during the rearing period is beneficial to enhance the use of three-dimensional space during the production period (Brantsæter et al., 2016a; Gunnarsson et al., 2000), and previous work has also shown that exposure to less complex environments led to impaired spatial cognition at 23 weeks of age (Tahamtani et al., 2015). These studies therefore suggest early environmental complexity can have lasting effects on hens' cognitive abilities, and these differences could be reflected in the individuals' hippocampal formation, which is involved in spatial cognition (see section 1.6).

1.4 Interactions between rearing and laying environments

As seen in the previous sections, the current environment can affect individuals' welfare and the early-life environment contributes to shaping physiology and behaviour through developmental plasticity. This developmental plasticity is thought to be adaptive and to be a way to enhance the fit between the individual and its environment.

Different theories have been developed to explain developmental plasticity and the interaction between early and adult environments. These theories are often used in life history studies and use the fitness of the individual as an outcome measure, but they could also be applied to other fields. Nettle and Bateson (2015) discussed the adaptive developmental plasticity (ADP) and distinguished between two possible theories: the informational ADP and the somatic state-based ADP. The informational ADP suggests that the environment experienced during the development of the individual gives an advantage in fitness if it actually forecasts the future environment. This is similar to what the Predictive Adaptive Response (PAR) hypothesis implies, with the early-life environment predicting the future environment and affecting the development of the individual to become adapted to their environment as well as possible (Bateson et al., 2014). Under this theory, having a mismatch between the predicted environment and the actual environment could have negative consequences for individual fitness (Bateson et al., 2014). In a more applied approach, the informational ADP theory suggests that housing laying hens in a poorer environment than the one they were reared in can be deleterious, as supported by the higher mortality rate of aviary-reared hens housed in furnished cages compared to cage-reared hens (Tahamtani et al., 2014). On the other hand, housing laying hens in a richer and more complex environment than the one they were reared in could also have negative consequences, e.g. if a higher level of fearfulness prohibits the birds from accessing resources or navigating successfully between the different tiers of the aviary. In contrast, the somatic state-based ADP theory suggests that the presence of some characteristics in the environment experienced during early-life shapes the phenotype in such a way that it always gives an advantage in fitness compared to individuals not exposed to these environmental characteristics (Nettle & Bateson, 2015). This implies that individuals experiencing a rich environment during early-life will have an advantage over individuals experiencing a poor environment, disregarding the quality of their current adult environment. This would

mean that hens reared in an aviary will be advantaged compared to cage-reared hens no matter to which environment they are transferred to for the production period.

Because of the clear division between the rearing period and the production phase, laying hens are very well suited to studying the interaction effects between early-life and adult environments. A review by Janczak and Riber (2015) suggests that the PAR applies, as housing hens in a different type of environment that they were reared in leads to welfare issues. But not all outcome measures seem to support the PAR theory, as hens reared in an aviary showed higher cognitive performances than cage-reared hens, despite being housed in furnished cages (Tahamtani et al., 2015). It could be argued, however, that having higher cognitive abilities when housed in a cage system is not adaptive, since it is not necessary to the individual survival and could induce costs (e. g., in terms of allocation of resources, or potential frustration). Higher cognitive abilities might nonetheless be adaptive when the individual has to face changes in its environment, therefore presenting an advantage compared to individuals with lower cognitive abilities.

1.5 Stress

As previously mentioned, stress is an important component of welfare. The life experienced by laying hens in the egg industry can be stressful, and if a stressor is applied over a long time and overtaxes the individual's ability to cope, it can eventually cause chronic stress in birds. In the literature, the word "stress" has been defined in different ways. In this thesis, "stress" will be used to describe a state of threatened homeostasis (Johnson et al., 1992). Homeostasis can be threatened by a variety of potential stressors and the response depends on different factors, including the individual's perception of the stressor. In chickens, a lack of environmental enrichment can be a source of stress by increasing competition for resources or not enabling the expression of some behaviours, such as exploration and foraging (EFSA Panel on Animal Health and Animal Welfare, 2023), thus causing frustration in birds.

1.5.1 Stress physiology

When facing a stressor, the individual's autonomic nervous system and hypothalamic–pituitary–adrenal (HPA) axis are mobilised (Barnett & Hemsworth, 1990). The former is responsible for the quickly activated "fight or flight" response, and acts by releasing adrenaline and noradrenaline into the blood stream (Blas, 2015). If exposure to the stressor lasts, the HPA axis gets activated. In chickens, the

HPA axis is functional from the first day post-hatching (Ericsson & Jensen, 2016). This is a well conserved neuroendocrine system among vertebrates, controlling the stress response (Romero & Gormally, 2019). Its activation ultimately results in the release of glucocorticoids, such as corticosterone in birds and rodents, into the blood stream. Briefly, exposure to a stressor leads to the activation of neural cells in the paraventricular nucleus, situated in the hypothalamus. These neurons produce and release two hormones, the corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT), the non-mammalian homolog of arginine vasopressin. Both CRH and AVT regulate the secretion of hormones in the pituitary gland. When they bind to their receptors located in the anterior lobe of the pituitary gland, they stimulate the secretion of the adrenocorticotropic hormone (ACTH). Once released into the bloodstream, the ACTH reaches the adrenal glands where the glucocorticoids are synthetized and secreted. Glucocorticoids are then involved in gluconeogenesis by muscle catabolism and mobilization of fatty acids from adipose tissue, which allows the organism to cope with challenges by mobilizing resources. Glucocorticoids then play a role in regulating the release of CRH and ACTH via a negative feedback system by binding to mineralocorticoids (MR) and glucocorticoid receptors (GR) in the paraventricular nucleus and the pituitary gland. (Herman et al., 2020; Kuenzel et al., 2020). The hippocampal formation (HF) also plays a role in regulating the HPA axis (see section 1.6).

1.5.2 Intensity and duration of the stressor

Stress affects the organism differently depending on the intensity and the duration of exposure to stressors and can be divided into two categories: *acute* stress, and *chronic* stress. Acute stress is brief, with a quick return to homeostasis. It is the organism's response to perceived threats that have a relatively short duration, as described in the previous section, and it is essential for survival (Boissy, 1995; Johnson et al., 1992). Though the activation of the HPA axis in response to an acute stressor is adaptive to protect the individual from potential dangers, prolonged activation as a consequence of long-term exposure to stressors can have deleterious effects on the individual. When exposure to the stressor is persistent, repeated or that the individual fails to return to homeostasis, it can cause chronic stress. Chronic stress is known to have negative effects on health and welfare, with induction of depression and depressive-like behaviours (McEwen, 2017). In calves and pigs, chronic stress led to a higher sensitivity to ACTH and disrupted feedback control, respectively (Désiré et al., 2002). It has also been shown to affect the immune system, leading to

immunosuppression and an increased sensitivity to pathogens in pigs and quails (Luo, Z. et al., 2020; Nazar & Marin, 2011). Similarly in chickens, repeated administration of corticosterone mimicking a state of chronic stress led to immunosuppression (Dohms & Metz, 1991). In addition, chronic stress affected cognition in rodents (Conrad, 2010) and in hens (Zidar et al., 2018), and decreased neurogenesis in the hippocampus (rodents: Alves et al., 2018; hens: Gualtieri et al., 2019). However, the time needed to induce a state of chronic stress should be carefully considered, as exposure to repeated stressors for only one week did not affect emotivity in quails, but stressed birds showed better performances in the reversal phase of cognitive testing (Calandreau et al., 2011).

1.5.3 Measuring stress

Acute and chronic stress can be measured both at the behavioural and at the physiological levels. A common approach to measure stress responsivity is to expose the individuals to an acute stressor (e.g., a restraint or exposure to a new environment) and to measure the levels of glucocorticoids in their plasma before and after exposure to the stressor (Mormède et al., 2007). The magnitude of the increase translates to the stress responsivity of the individual. A high sensitivity and a strong response to stressors is not adaptative and potentially leads to disproportionate fear responses and to frequent and exaggerated activation of the HPA axis. Looking at the plasma concentration before exposure to the stressor can also give information on the baseline level of stress of the individual, and chronic stress can be reflected by a prolonged increase in glucocorticoid levels (Herman, 2022). However, not all studies report elevated baseline levels of corticosterone in chronically stressed birds (Gormally & Romero, 2018; Gualtieri et al., 2019), which makes chronic stress harder to detect and measure. In addition, measures of corticosterone concentration in plasma are limited to one point in time, which might not reflect the overall state of the individual (Herman, 2022). This emphasizes the need to use different markers of stress to get a comprehensive overview of the birds' state. One alternative to plasma concentration of corticosterone is to study its concentration in feathers. As corticosterone is deposited in birds' feathers during growth, this can give an overview of their stress response over a certain period of time. However, aversive stimuli are not the only factors that can affect the activation of the HPA axis, as physical exercise or circadian rhythm also influence the secretion of glucocorticoids (Mendl et al., 2009; Schoenfeld & Gould, 2012). Measuring corticosterone concentration in feathers might therefore lead to false conclusions, as exposure to stressors of different valences leads

to the same outcome. The heterophil:lymphocyte (H:L) ratio has therefore been used as a way to measure the long-term stress experience of the individual (Maxwell & Robertson, 1998). H:L ratio is known to increase under stressful conditions, such as heat stress or transportation (Maxwell, 1993). This measure however only allows for the assessment of the individual negative experience and does not allow to evaluate potential positive experiences. Adult neurogenesis has recently been validated as a measure of cumulative affective experience in the mammalian hippocampus (Poirier et al., 2019) and in the avian hippocampal formation (Armstrong, 2020). Exposure to changes in glucocorticoid levels affects neurogenesis differently depending on the valence of the stimuli (Schoenfeld & Gould, 2012), making it very suitable to assess the individual experience. The hippocampal formation and its role in cognition and stress regulation will be described in the next section.

1.6 Hippocampal Formation

In mammals, the hippocampus is known to be involved in spatial cognition and the regulation of the HPA axis activity (Jacobson & Sapolsky, 1991). Lesions or ablation of the hippocampus lead to an increased basal concentration of glucocorticoids (Jacobson & Sapolsky, 1991), a higher increase following exposure to a stressor (Herman et al., 1998), and impaired performances in a detour test in rats (Thompson et al., 1984). The mammalian hippocampus can be divided into two subregions along the longitudinal axis, with the septal part (dorsal in rodents, posterior in primates) being primarily involved in spatial cognition and the temporal part (ventral in rodents, anterior in primates) in the regulation of the HPA axis (Moser & Moser, 1998). However, the dorsal part of the rodent hippocampus seems to also be involved in the regulation of circadian secretion of glucocorticoids, as lesions targeted at the ventral part do not affect it (Herman & Mueller, 2006). The hippocampal formation (HF) of birds is thought to be homologous to the mammalian hippocampus, with the rostral pole being involved in spatial cognition and the caudal pole in regulating the stress response (Smulders, 2017).

The regulation of the HPA axis by the telencephalon in birds has recently been reviewed by Smulders (2021). Briefly, the HF is involved in the negative feedback system allowing hormone levels to return to baseline after an acute stressor and in regulating circadian secretion of glucocorticoids. This is made possible by the large number of GR and MR expressed in the HF. MRs have a higher affinity to glucocorticoids than GRs and are essentially involved in the circadian regulation of

glucocorticoids levels. GRs having a lower affinity to glucocorticoids, they are primarily involved in the negative feedback system regulating the stress response (Herman et al., 2020). Besides being involved in the regulation of the HPA axis activity, the HF is sensitive to stress. Exposure to chronic stress led to a reduction of MR receptors in the HF of wild starlings, which could be a source of alteration of the HPA-axis functioning (Dickens et al., 2009). The same effect of chronic stress on MR receptors has also been shown in quails (Zimmer & Spencer, 2014). This resulted in birds which were less fearful, but no effects on corticosterone concentrations were found, implicating other mechanisms behind this difference in fearfulness (Zimmer et al., 2013). In addition to effects on MR and GR expression, stress can affect neurogenesis and neural plasticity in the HF by altering cell proliferation, differentiation, and survival. Among other markers and methods, it is possible to assess hippocampal plasticity by studying the density of cells expressing doublecortin (DCX). DCX is a microtubule-associated protein expressed by young migrating neurons (Francis et al., 1999). Studying DCX+ cell density is therefore a way to quantify migration and differentiation of new neurons in the HF. In birds, however, DCX as a marker of neurogenesis is still debated as it could also be expressed by neurons with high plasticity (Vellema et al., 2014). In a conservative approach, I will refer to hippocampal plasticity rather than neurogenesis when referring to DCX+ cells in the avian brain. DCX+ cells can display different morphologies and be divided in two main groups: bipolar and multipolar. Cells from the former group are thought to be younger and still migrating, while multipolar cells are thought to be more mature and settling. Distinguishing between these two categories allows for the study of effects on DCX+ cell survival.

As previously mentioned, hippocampal plasticity is affected differently depending on the valence of the experience. In rodents, hippocampal neurogenesis is downregulated by chronic stress (Alves et al., 2018; Yun et al., 2010). Similarly in laying hens, exposure to unpredictable mild stress or keel bone fracture severity downregulated neural plasticity (Armstrong et al., 2020; Gualtieri et al., 2019). Conversely, exposure to positive experiences, such as physical exercise and enrichment, increased neurogenesis in rodents (Olson et al., 2006; van Praag et al., 2000) and birds (Melleu et al., 2016). Both the downregulation due to negative experiences and the upregulation due to positive ones rely on higher glucocorticoid titres (Lehmann et al., 2013). Stimulation of hippocampal neurogenesis in rodents is often associated with better cognitive abilities, as shown by positive effects of exercise or enrichment on solving cognitive tasks (Fabel et al., 2009; Lafenetre et al.,

2010; van Praag et al., 2000). There is also a relationship between spatial cognition and hippocampal plasticity in food-hoarding birds, with individuals not allowed to store and retrieve food showing lower levels of plasticity than the ones able to perform these behaviours (LaDage et al., 2010; Patel et al., 1997). Impairment of spatial cognition and changes in HPA axis activity due to chronic stress could therefore partly be due to effects on hippocampal plasticity.

1.7 Knowledge gaps

We saw in the previous sections that the early-life environment is a strong driver in the development of an individual and influences its subsequent life, including fearfulness, cognition and stress sensitivity. Despite these well-known effects, chicks are still reared in a large variety of environments, which leads to birds with different behavioural and physiological phenotypes. Previous studies have investigated the effects of rearing in cages or in an aviary on fearfulness (Brantsæter et al., 2016a; Brantsæter et al., 2016b; Brantsæter et al., 2017) or cognitive abilities (Tahamtani et al., 2015) at the onset of lay, but longer-term studies are lacking. The majority of studies focused on the few weeks following transfer to the laying farm, which is not sufficient to give a comprehensive view and deep understanding of the hens' experience. It is also less documented whether the adult environment affects the hens differently based on their early-life experience. This thesis thus aims at investigating the long-term effects of different rearing environments in addition to the medium-term effects, and to test whether early and adult environments interact in shaping the phenotype of the individual with regards to spatial cognition, fearfulness and stress responsivity. Furthermore, the extent to which environmental complexity during rearing can affect hippocampal plasticity throughout the hens' life is also under investigated, and so are the effects of environmental enrichment during adulthood. I therefore aimed at understanding how rearing and laying environments complexity would interact and influence neural plasticity on the long-term, if the effects were specific to HF subregion and whether behavioural traits believed to be influenced by cognitive abilities and/or stress and fear would be associated with hippocampal plasticity.

2 Aims and objectives

The aim of this thesis was to get a deeper understanding of the medium- and long-term effects of environmental complexity during rearing on several traits important to the welfare and development of laying hens, and how the provision of enrichment during the production period would influence these effects. This study particularly focuses on fearfulness, stress responsivity, cognitive abilities, and neural plasticity. The project was divided into the following objectives:

- **Medium- and long-term effects of the environmental complexity during rearing.**
  The aim was to assess whether hens reared in cages or in an aviary would show different levels of fearfulness (**Paper I**) and cognitive abilities (**Paper II** and **III**). Long term effects on stress responsivity (**Paper IV**) and hippocampal plasticity (**Paper III**) were also investigated.
  It was predicted that hens reared in the aviary would be less fearful than cage-reared hens, have a lower stress responsivity and better cognitive abilities. It was also predicted that aviary-reared hens would show a higher degree of hippocampal plasticity than cage-reared hens.

- **Effects of environmental enrichment provision during the production period and interaction with the rearing environment.**
  The second aim of this thesis was to understand whether the provision of additional enrichment in the cage during the production period reduced fearfulness and stress responsivity in hens reared in cages or in an aviary (**Paper I** and **IV**). We also aimed at understanding whether hens housed in an enriched environment during the laying phase had better spatial cognition than hens housed in standard cages, and how this related to levels of neural plasticity in the hippocampal formation (**Paper III**).
  It was predicted that additional enrichment in the cage would reduce stress responsivity and decrease fearfulness, as well as enhancing hippocampal plasticity and cognitive abilities.

3 Materials and Methods

This thesis aimed at studying the medium-term and long-term effects of the rearing environment, and to study the interaction of the rearing environment with the laying environment. The effects of environmental complexity during rearing were studied in all papers, while the effects of enrichment during the laying phase were investigated in all papers except **Paper II**. Methods used will briefly be developed in the next sections, with more details available in papers **I**-**IV**.

3.1 Animals and housing conditions

3.1.1 Experimental animals

All the hens used in this thesis were non beak trimmed White Leghorn coming from the same rearing farm and then housed at the same production farm. Medium-term effects of the rearing environment complexity were studied on hens up to 40 weeks of age (**Paper I** and **II**), while the long-term effects of the rearing environment and effects of adult enrichment were studied on hens aged from 52 up to 65 weeks of age (**Paper I**, **III** and **IV**). Chicks were divided into cage-reared and aviary-reared groups at the rearing farm and were then housed in standard furnished cages (Victorsson T10, see details in 2.1.3) or additionally enriched furnished cages at the production farm. Hens reared in the aviary (a) or cage (c) and housed in standard furnished cages (S) will be referred to as aS and cS, respectively. Following the same pattern, hens housed in enriched furnished cages (E) will be referred to as aE and cE. An overview of the distribution of the hens between the different housings and experimental timeline is shown in Figure 2. The birds tested to study the long-term effects of the rearing environment came from different cages than the birds tested to study the medium-term effects of the rearing environment. All the experimental work took place in 2020 at the production farm (Moer Gård, Ås, Norway) and procedures were approved by the Norwegian Food Safety Authority (FOTS ID 22443).

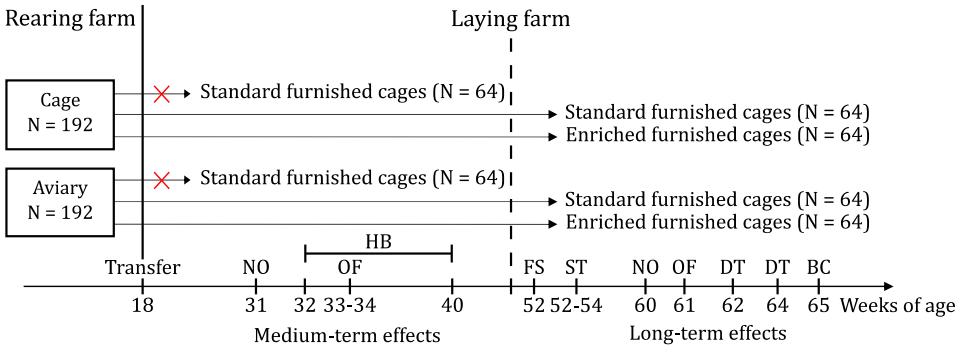

Figure 2: Distribution of individuals in the different types of housing. Pullets were transferred from the rearing farm to the production farm at 18 weeks of age. A subset of bird was tested at 30–40 weeks of age (**Paper I** and **II**) and the rest at 52-65 weeks of age (**Paper I**, **III** and **IV**). The red crosses indicate that one hen was removed from the cages at 23 weeks of age as part of another experiment. NO: Novel object test; OF: Open field test; HB: Holeboard test (including the habituation phase); FS: Feather scoring; ST: Restraint stress test; DT: Detour test; BC: Brain collection.

3.1.2 Rearing conditions

Hens were reared from one day of age at the same commercial rearing farm. All chicks were housed in the same room containing 38,000 chicks in total in a raised Natura primus 16 aviary system (www.bigdutchman.com, see Figure 3) until they reached 18 weeks of age. To implement different levels of complexity at the rearing farm, a subset of chicks (N = 250) was kept within a tier of the aviary to simulate cage rearing while the rest of the chicks had access to the full room from five weeks of age. Regardless of the rearing condition, day-old chicks were confined in the aviary row until they reached 3-5 weeks of age, as advised by the breeder's recommendations. They had access to feed, water and perches inside the aviary row and the mesh floor of the row was lined with chick paper. Starting at three weeks of age, when big enough to reach the different tiers by themselves, the chicks from the aviary reared treatment started to be released in the aviary room. The front of the cages from the first (bottom) tier were opened at 3 weeks of age, and the front of the cages from the second (middle) tier were opened at 4 and 5 weeks of age. Chicks from the cage-reared treatment stayed confined inside the aviary row for the whole duration of the rearing period. Standard procedures were otherwise in accordance with the breeder's guidelines.

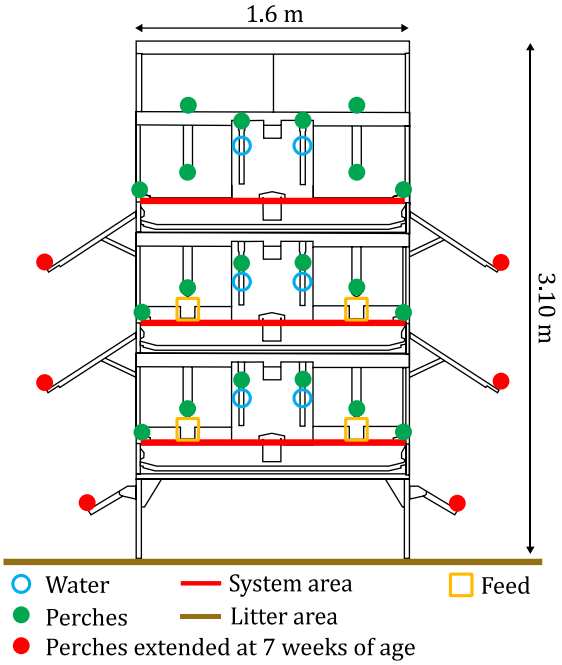

Figure 3: Schematic representation of Natura Primus 1600 viewed from the end of the row showing feed lines, water lines, and perches (based on the Big Dutchman leaflet).

3.1.3 Housing during laying

At 18 weeks of age, 192 hens from each rearing treatment were transported from the rearing facilities to the experimental farm. The hens were allocated to two different types of housing, balanced across the two rearing treatments: standard furnished cages (N = 256) or additionally enriched furnished cages (N = 128). All hens were housed in social groups of four from the same rearing condition. In the set of cages used to study the effects of the medium-term effects of the rearing environment, one hen per cage was removed at 23 weeks of age as part of another experiment. The hens in these cages were therefore housed in social groups of three from that age on. Each experimental cage consisted of two Victorsson T10 cages adjoined by an opening. These pairs of cages containing four hens are hereafter referred to as a cage. Each cage measured 240 cm x 80 cm x 60 cm (width x height x depth) and was furnished with two nest boxes, four perches and two dustbathing platforms on the roof of each nest box (Figure 4 A). To increase environmental complexity and study the effect of adult enrichment, 128 hens were housed in additionally enriched cages (Figures 4 B

and 5). Enriched cages were the same as standard furnished cages with the addition of a dustbathing platform, a hemp pompon and some polyethylene curtains (Figures 4 B and 5). The hemp pompon was provided as an enrichment the hens could peck at. Curtains made of a polyethylene tarp were hung below one of the perch and above the opening between the two parts of the cage to increase the structural complexity of the cage environment. The dustbathing platform was refilled weekly with a mix of feed and dustbathing pellets. To slightly increase the environmental variability, the position of the dustbathing platform and pompon was changed to the other side of the cage every other month, starting from 42 weeks of age. The distribution of hens in the henhouse was balanced so that hens reared in cages were always housed in cages next to hens reared in the aviary.

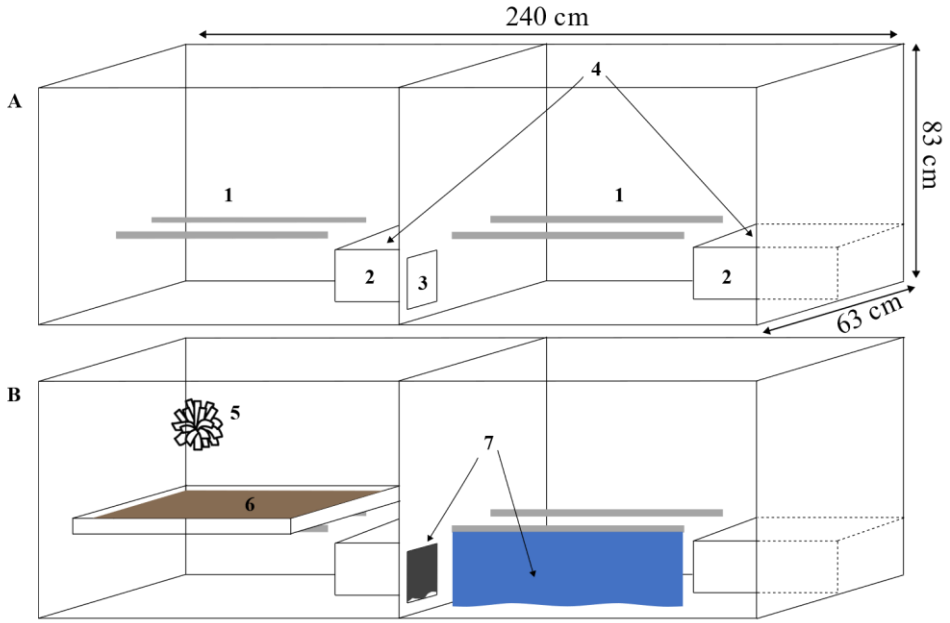

Figure 4: Schematic representations of a standard cage (**A**) and an additionally cage (**B**), three-quarter front view, showing (**1**) the perches, (**2**) the nest boxes, (**3**) the opening between the two parts of the cage, (**4**) the dustbathing trays, (**5**) the hemp pompom, (**6**) the additional dustbathing tray and (**7**) the curtains. The features 1-4 were also accessible in the additionally enriched cages.

Figure 5: Picture showing the additional enrichment provided in the enriched furnished cages used in **Paper I**, **III** and **IV**.

3.2 Behavioural tests

All behavioural tests, with the exception of the novel object test, were performed in two temporary arenas built in the henhouse (Figure 6). The arenas measured 177 x 350 cm with 190 cm high walls. Three of the walls of each arena were made of wood frames covered by a dark tarp, the fourth wall being the wall of the henhouse in concrete, and the floor was covered with particle board panels. Cameras (Axis m1124-e network camera, Noldus, The Netherlands) mounted on the wall of the hen house were used to record the tests using the MediaRecorder system (Noldus Information Technology, Wageningen, The Netherlands).

3.2.1 Fearfulness tests

Fearfulness was assessed at the group level by testing the hens in a novel object test in their home cage and at the individual level by an open field test (**Paper I**). Each test was performed on a subset of hens at 31-34 weeks of age to study the medium-term effects of the rearing environment and at 60-61 weeks of age to study the long-term effects of the rearing environment and the effects of environmental enrichment.

Figure 6: Photo showing the two arenas during the holeboard test (**Paper II**).

3.2.1.1 Novel object

The novel object used was a small pink egg cup glued on a plywood square (19 cm x 19 cm) baited with mealworms (*Tenebrio molitor*), which looks like nothing the hens might have encountered previously. Right before introducing the novel object in the cage, the hens were moved to one side of the cage. The novel object was placed on the opposite side of the cage (see Figure 7), and the observer moved as far away as possible from the cage (~1.5 m) to score the test for 5 min. Initially, only the latency for the first bird to peck at the cup and the number of pecks to the cup were recorded. However, considering the low occurrence of pecking observed during the first trials, the latency to enter the side of the cage holding the novel object was added to the behaviour recorded. This lowered the number of cages used in the analysis to 9 cages of cage-reared birds and 10 cages of aviary-reared birds for the first round of testing. For the second round of testing, 16 cages from each group were tested. In cages with additional enrichment, the curtain hung above the opening between the two parts of the cage was removed prior to the test to ensure the novel object was visible by the hens.

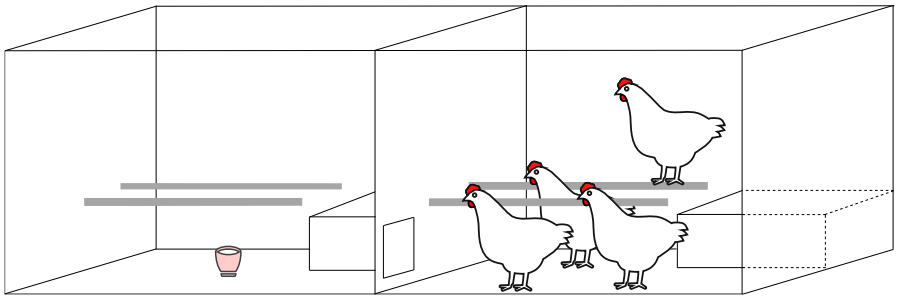

Figure 7: Schematic representation of the novel object test. The four hens were moved to one side of the cage prior testing, and the novel object was placed on the other half of the cage.

3.2.1.2 Open field

Two birds per cage were selected to be tested in an open field test at 33-34 (cS: N = 30; aS: N = 30) and 61 (cS: N = 30; cE: N = 31; aS: N = 30; aE: N = 32) weeks of age. The test lasted for 10 min and was video recorded for further analysis. For the first round of testing, the hens were manually released in a corner of the arena by the experimenter. For the second round of testing, start boxes which could be lifted from the outside of the arenas were used to synchronise the start of the tests. The latency to leave the start area and the total distance walked in the arena were measured using EthoVision X9 (Noldus Information Technology, Wageningen, The Netherlands). To prevent an overestimation of the distance walked, the track of each hen was smoothened and its central point was fixed to the same location until the distance moved was at least 5 cm.

3.2.2 Cognitive tests

In **Papers II** and **III**, we investigated the effects of environmental complexity on spatial cognition. **Paper II** focused on the medium-term effects of the rearing environment on the performances in a holeboard test, while **Paper III** focused on the long-term effects of the rearing environment and the provision of enrichment during the production period on the ability to perform a detour. All birds tested in the cognitive tests were previously tested in the novel object and open-field tests described above (**Paper I**).

3.2.2.1 Holeboard test

Two hens per cage were selected at 32 weeks of age to be habituated to the holeboard test (**Paper II**). This test was used to assess the spatial cognition of the hens and consisted of four phases (uncued, cued, over-training and reversal) during which the hen had to find three baited cups among the eight distributed in a 4x2 matrix (see Figure 8). Prior to the testing phases, the hens were habituated to the cups and the arena. Habituation to the cups lasted for 5 days, during which the hens were exposed to the cups baited with three mealworms (*Tenebrio molitor*) in their home cage or in the feed trough. Habituation to the cups was followed by 5 days of habituation to the arena, during which all cups were baited to encourage exploration of the arena. At the end of the habituation period, one hen per cage was selected for the testing phase based on the number of worms eaten in the habituation sessions. Two cages per group were excluded from the testing phases due to the low number of worms eaten by both hens.

For all phases of the test, only three cups out of the eight were baited with one mealworm. For the three first phases, the configurations of baits in the arena remained unchanged. Visual cues were added to the baited cups during the cued phase and the arena was returned to its uncued version for the over-training phase. During the reversal phase, the configuration of baits was changed to investigate cognitive flexibility. For each trial, the numbers of visits to the baited and non-baited cups were recorded and ratios were calculated to estimate different memory parameters:

- **Working Memory Ratio (WM):** Shows the capacity to avoid revisiting baited cups that have already been visited.

$$\frac{\text{Number of rewarded visits}}{\text{Total number of visits to the baited cups}}$$

- **General Working Memory Ratio (GWM):** Shows the capacity to avoid revisiting cups that have already been visited.

$$\frac{\text{Number of different cups visited}}{\text{Total number of visits}}$$

- **Reference Memory Ratio (RM):** Reflects the ability to discriminate between baited and unbaited cups.

$$\frac{\text{Total number of visits to the baited cups}}{\text{Total number of visits to all cups}}$$

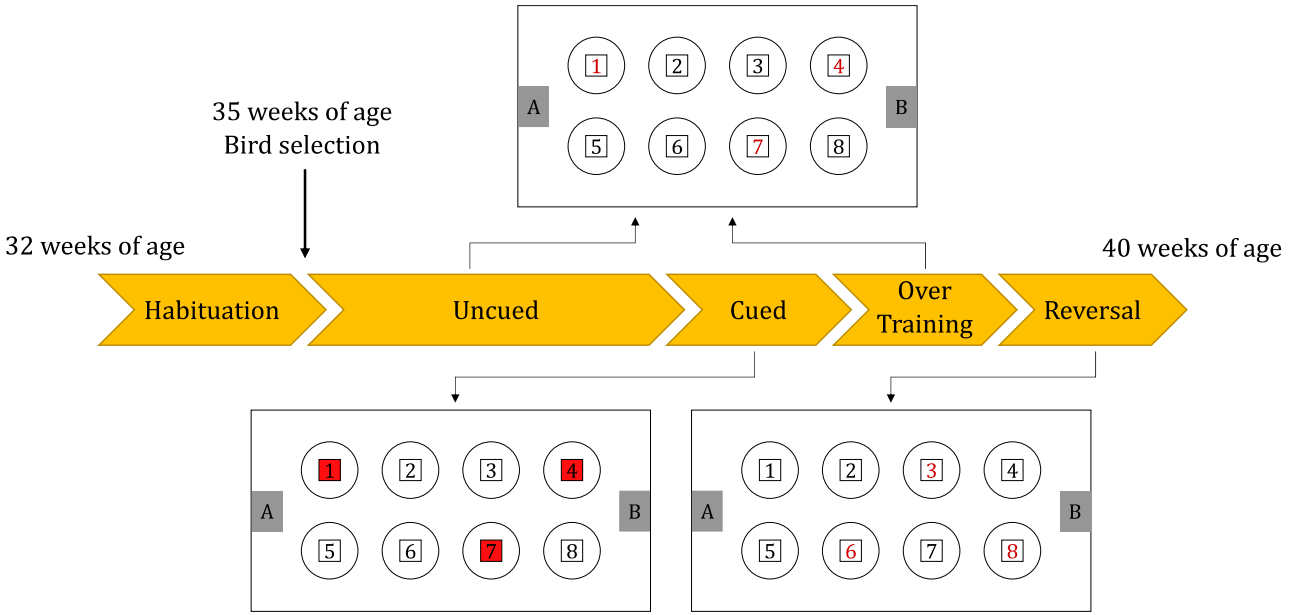

Figure 8: Timeline of the holeboard test with a schematic representation of the arena viewed from above. A and B grey boxes represent the two possible start points of the trial. An example of reward configuration is shown in red on each drawing. The Uncued phase lasted 12 days, the cued phase 4 days, the over-training phase 4 days and the reversal phase 5 days. The birds were tested twice a day for the whole test, except on the first day of the uncued phase (only one trial).

The latency to find the first bait and the trial duration were also recorded for each trial. Trial duration was defined as the time elapsed to find all baits, or the maximum trial duration of 5 min, whichever occurred first. Latencies and memory ratios were averaged per blocks of two consecutive days for the analysis. The reversal phase consisting of five days, the first two days and last two days were averaged, and the third day of that phase was averaged on its own (two trials).

3.2.2.2 Detour test

We tested 64 laying hens in a detour task (**Paper III**) to assess their spatial cognitive abilities. These hens were cage- or aviary-reared, and then housed in standard or enriched furnished cages during the laying period (4 groups, n=16 per group). For the test, a hen was placed in a detour compartment with solid side walls, an open rear end and a grid front (Figure 9). It was facing a stimulus compartment containing two familiar hens and food as a motivation to make the detour. Each hen was tested twice (at 62 & 64 weeks) and latencies to perform the detour were recorded, with a cut-off of 10 minutes. The detour was considered performed when the central point of the hen crossed the dotted line (Figure 9).

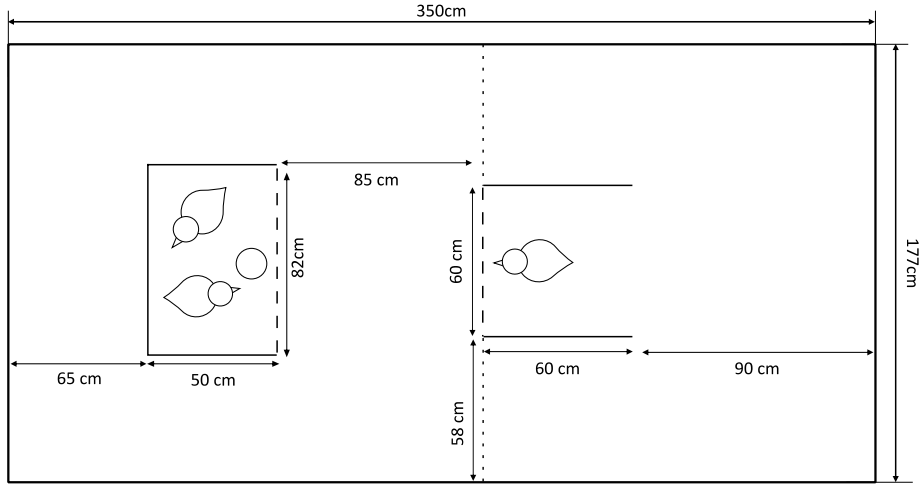

Figure 9: Schematic representation of the detour arena, viewed from above. The stimulus compartment can be seen on the left-hand side of the drawing, and the detour compartment on the right-hand side. The detour was considered done when the hen crossed the thin dotted line.

3.3 Plumage assessment

At 52 weeks of age, the plumage quality of the birds was visually assessed using the Welfare Quality Assessment protocol for poultry. The head and neck, back and rump, and belly areas were scored. Scores ranged from 0, being an almost perfect plumage condition, to 2, meaning a poor plumage condition with a naked area of at least 5 cm at its widest.

3.4 Laboratory methods

To assess the cumulative experience of stress and the stress responsivity of the hens, we performed a restraint stress test (**Paper IV**) and analysis of hippocampal plasticity (**Paper III**). Hens used in the analysis of hippocampal plasticity were previously tested in the novel object test, the open field test and the detour test (**Paper I** and **III**).

3.4.1 Blood sampling and analysis

At 52-54 weeks of age, hens were tested in a restraint stress test (**Paper IV**). Two hens per cage (one control, one stressed hen) were consecutively taken out of their home cage and a blood sample was collected from their wing vein. The second hen sampled was taken out of the cage once the sample from the first hen was collected. Samples were taken within 3 min following capture, which allows to get a baseline corticosterone concentration (Romero & Reed, 2005). After blood collection, the control hen was returned to its home cage while the stressed hen was exposed to a restraint stress. The hen was placed and suspended in a small mesh laundry bag for 10 min, which is enough time to induce a stress response (Ericsson et al., 2016). Blood samples were collected from the other wing vein after 12 min for both hens. The treatment of the first hen taken out of the cage (stressed or control) and sampled first was balanced across housing treatments.

Plasma was separated from the red blood cells by centrifugation and kept at -80°C until further analysis. Samples were shipped to the University of Veterinary Medicine in Vienna and corticosterone levels in the plasma were analysed in collaboration with Rupert Palme. Half a millilitre of plasma was extracted with 5 ml diethylether, dried down and re-dissolved in a similar amount of EIA buffer. An aliquot of 50 µl of these extracts was analysed in an in-house corticosterone EIA (Palme, 1997).

3.4.2 Hippocampal plasticity

The brains of 12 hens per treatment group were collected at the end of the experimental work (**Paper III**). At 65 weeks of age, hens were anesthetised and killed by cervical dislocation. One brain hemisphere was kept for immunohistochemistry, while the Hippocampal Formation (HF) of the other one was dissected to be used for molecular analysis (results not reported in this thesis). Left and right hemispheres collected for immunohistochemistry were balanced within each treatment group.

Brain samples used for immunohistochemistry were fixed in 4% paraformaldehyde for 24 h before being cryoprotected in 30% sucrose solution for 48 h. The sucrose solution was renewed once during this time span. Brain samples were then embedded in OCT and stored at -80°C. Coronal sections (50 µm thick) were cut and stored at -20°C in cryoprotectant (30% glycerol and 30% ethylene glycol in 0.1 M PBS). Using a free-floating section protocol, the sections were stained by immunofluorescence against doublecortin. Sections were pre-treated with a 30 min endogenous peroxidase inhibition (3.3% H_2O_2) for 30 min and in a blocking solution (2% goat serum, 1% Bovine Serum Albumin in 0.1 M PBS containing 0.3% Triton X-100.) for 60 min. They were then incubated for 16 hours at 4°C in a 1:1000 polyclonal antibody raised in rabbit against DCX solution (Abcam ab18723), followed by a 120 min incubation in a 1:2000 goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody solution (Alexa Fluor Plus 594- RED) at room temperature. Sections were then sorted along the rostro-caudal axis and mounted on gelatine subbed slides. Nuclei were stained with a working solution containing 300nM DAPI in 0.1M PBS. For 3 of the brains, the nuclei were DAPI stained after being mounted on the slides. For all other brains, staining with DAPI was done directly into the wells, before sorting and mounting. All slides were coverslipped with ProLong glass antifade mountant (Fisher, ref. P36984). Between each step of the protocol, sections were washed (3 x 5 min) in 0.1M PBS, except after the blocking solution where they were quickly rinsed in dH_2O before being incubating with the primary antibody.

Two rostral sections spaced by 800 µm (interaural 3.76-2.32, Puelles et al., 2007) and one caudal section (beyond interaural 0.80) per brain were analysed. The HF of each section was outlined at x10 using Zeiss AxioImager with Apotome systems and the Zen software. Tiles measuring 332.8 µm x 332.8 µm spaced by 75 % were imaged at x40 magnification, with a total of 19 Z-stacks spaced by 0.90 µm. Images from 6 brains per treatment group were analysed with the Fiji software (Schindelin et al., 2012),

using the cell counter plug-in. For each tile, the tissue area was measured and cells expressing doublecortin were counted. Cells were split in two categories: bipolar and multipolar (Figure 10). The bipolar cells were defined as small/medium elongated cells with a maximum of two processes. The multipolar cells were defined as medium/large cells with at least three processes.

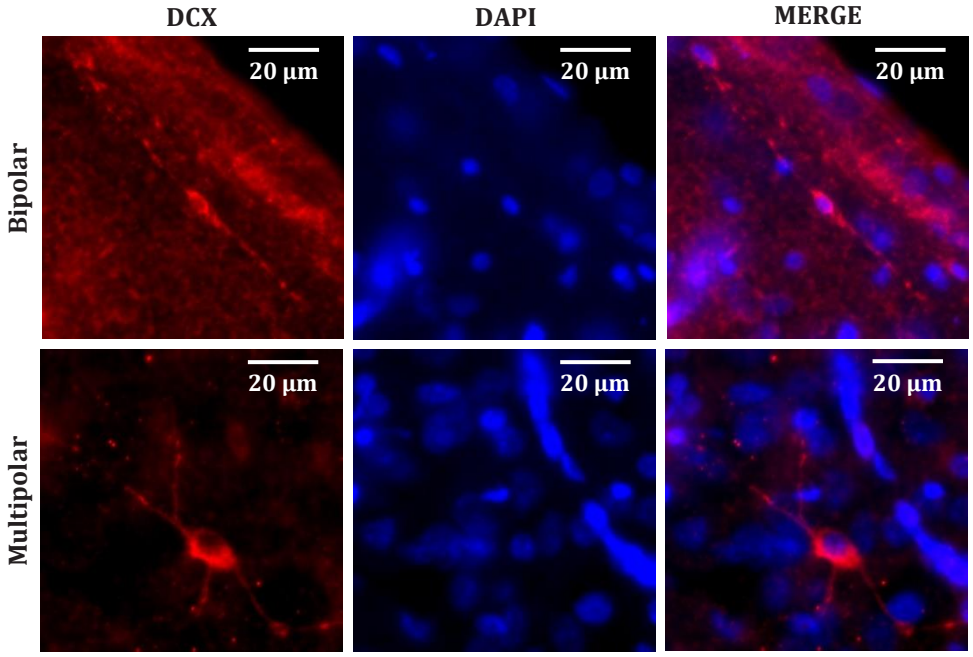

Figure 10: Representative images of a bipolar and a multipolar DCX+ cell taken at x40 magnification.

3.5 Statistical analyses

All statistical analysis were performed using the R software (R Core Team, 2022). Statistical approaches used during this thesis were linear (mixed-effects) models (L(M)M), generalised linear (mixed-effects) models (GL(M)M), chi-square tests, accelerated failure time models, t-tests, Pearson correlations and ordinal logistic regression models. L(M)Ms were visually examined and complied with the assumptions for normality of residuals and homogeneity of variances.

3.5.1 Medium-term effects of the rearing environment

In **Paper I**, the latency to approach the novel object was analysed with a LM while the variables from the open field test were analysed with LMMs. In both models, the rearing environment was used as a predictor. For the open field test, the Cage ID was used as a random effect to account for the lack of independence induced by testing two hens per cage. Despite transformation, the distance walked in the arena did not fulfil the homogeneity of variance criteria. The values were thus averaged per cage, and a Welch t-test for data with non-homogeneous variances was used.

For the holeboard test (**Paper II**), each phase of the holeboard was analysed separately and the transitions between each phase were also analysed with LMMs. The memory ratios or latencies studied were used as response, and the rearing environment and trial blocks were used as a predictor with the Hen ID as a random effect to account for repeated measures. Data from the habituation phase were analysed with GLMMs with the number of worms eaten per day as the response. The rearing environment, habituation day and whether the hen was selected or not for the test phases were used as predictors. Two-way interactions between the predictors were included in the model, and the Hen ID nested within the Cage ID was used as a random effect.

3.5.2 Long-term effects of the rearing environment and adult enrichment

For **Paper I**, the same approach as described in 3.5.1 for the medium-term effects of the rearing environment was used. The adult environment (enriched or not) and the interaction with the rearing environment were added in the models.

In **Paper III**, considering the high proportion of birds not completing the detour before the tests cut-off, latencies to perform the detour were analysed with an accelerated failure time model. The rearing environment, adult environment and round of testing were included as predictors. The Hen ID was included in the model as a cluster to account for repeated measures. This model was then expanded to include the distance walked during the open field test to study the relationship between the detour performance and the hens' fearfulness. Regarding HF plasticity, Pearson correlations were used to analyse cell densities between the two HF subregions and the two cell types. Differences in cell densities between the rostral and caudal HF and between cell types (bipolar or multipolar) were analysed

separately with a linear mixed effect model (LMM). To study the effects of the environment, one LMM per cell type (bipolar or multipolar) was run. The rearing environment, adult environment and HF subregion were used as predictors, with the Brain ID nested within the staining batch ID as random effects. To study differences between hens making the detour or not, another model with the same random effect structure was run with the HF subregion and whether the bird performed the detour or not on their first exposure to the test. As the HF is sensitive to emotional stimuli, we ran a similar model with the distance walked in the open field arena instead of the detour performance as a measure of the hens' fearfulness to test the relationship between fearfulness and hippocampal plasticity. Three-way and two-way interactions were included in the models.

In **Paper IV**, the plasma corticosterone concentration was analysed with a LMM. The rearing environment, adult environment, treatment (stress or control) and time (first and second samples) were used as predictors. Every two-way interaction was included in the model. The time to take the first sample counted from the first opening of the cage was also added as a predictor. The Hen ID nested within the Cage ID and the Plate ID were used as random effects. An ordinal logistic regression was used to analyse the data from the birds' plumage assessment. Each body area was analysed separately, with the rearing environment, adult environment and their interaction as fixed effects. The proportional odds assumption for the belly area was not fulfilled. Hence, the sum of the four hens sharing a same cage was calculated and analysed with a LM.

3.6 Ethical statement

Experiments performed during this thesis were approved by the Norwegian Food Safety Authority (Mattilsynet) under the FOTS ID 22443. During the planning stages of the experiment, the 3Rs guidelines were taken into consideration. Since the aim of this project was to assess the effects of environmental complexity at different life stages on the development and characteristics of laying hens, replacing animals was not a suitable option. In particular, it would have made it impossible to study the behaviour and physiology of insentient material. However, particular attention was allocated to calculating group sizes and refining the methods used. Group size was calculated to ensure the availability of enough statistical units to get a decent statistical power. The calculations were based on a power of 0.9 with an effect size ranging from 1.2 to 1.4 standard deviations. Though having extremely large sample

sizes is not ethical and should be avoided, a too small sample size is also not advised as it will make it less reliable to generalise the results from the experiments. For each axis of the project, the least invasive techniques were used. All animals were housed in environments which were at least fulfilling legal requirements, when not better, and monitoring and handling was always gentle. Behavioural tests used were not invasive and might only have induced slight discomfort to the birds in the form of moderate stress. More invasive methods such as blood or brain sampling were designed to induce as little discomfort as possible. Hens were tested only once in the restraint stress test and the minimum amount of blood necessary was sampled. Regarding brain sampling, birds were anesthetised before being killed to reduce the amount of stress and pain due to the procedure. The use of hippocampal plasticity as a marker of chronic stress can be subject to criticism as it requires the death of the animal, but as developed in sections 1.5.3 and 1.6 this measure gives a unique insight into the cumulative experience of the individuals. In addition, the marker of plasticity used (DCX) is an endogenous marker, which means no injections prior to euthanasia were needed to use it, which contributed to the refinement of methods.

4 Summary of papers

4.1 Paper I

The aim of this paper was to assess the effects of environmental complexity during rearing (cage or aviary) and the production period (standard or additionally enriched furnished cages) on laying hens' fearfulness. Naïve hens housed in standard furnished cages were tested in a novel object and an open field test at 30-33 weeks of age to study medium-term effects of the rearing environment complexity. Long-term effects were assessed at 60-61 weeks of age. Hens reared in cages or in an aviary and housed in standard or additionally enriched furnished cages were tested in a novel object and an open field test. The results showed the rearing environment complexity had limited effects on fearfulness. Hens reared in cages or in an aviary did not differ significantly in their latencies to approach the novel object or to leave the start arena in the open field test and in the distance walked in the arena. The same results were observed for the long-term effects of the rearing environment. The provision of enrichment during the production phase decreased the latency to approach a novel object but had no effect on the latency to leave the start area nor the distance walked in the open field test.

4.2 Paper II

Paper II tested the medium-term effects of environmental complexity during rearing on laying hens' spatial cognition. Over time, hens from both treatment groups became faster to find the baits and increased their memory ratios. The results showed an effect of the rearing environmental complexity during rearing on the latency to find the first bait for all phases of the holeboard test, except the reversal phase, and for the transitions between the uncued and cued phases, and between the cued and over-training phases. Aviary-reared hens were faster at completing the test and they started the cued phase with a higher reference memory ratio than cage-reared hens. Differences between the two rearing groups were also observed during the habituation phase, with cage-reared hens eating fewer worms across habituation days than aviary-reared hens.

4.3 Paper III

The aim of **Paper III** was to study the long-term effects of the rearing environmental complexity on spatial cognition and hippocampal plasticity, and the effects of adult enrichment. Results from the detour test showed that hens reared in cages were slower and less likely to perform the detour than aviary-reared hens. The likeliness to perform the detour was also associated with the distance walked in the open field, with hens walking less during the test being less likely to perform the detour. Adding the distance walked in the open field reduced the significance of the rearing environment. This suggests the two share some information despite the fact that no significant differences were observed in **Paper I** between hens from the two rearing environments. The provision of enrichment during the production period did not affect the performance during the detour test. The hens were also faster and more likely to perform the detour during their second exposure to the test, regardless of the different housings.

There were strong positive correlations between the cell density in the rostral and the caudal HF, and between the bipolar and multipolar cell types. Overall, there was a higher bipolar cell density than multipolar cell density, but there were no differences in the cell density between the two HF subregions. There were no significant effects of the rearing and adult environments on the bipolar cell density for either of the HF subregions. However, there was a significant three-way interaction between the rearing and adult environments and the HF subregion. Though pair comparisons were not significant, the provision of enrichment during the production period increased the multipolar cell density in the caudal HF for aviary-reared hens, while the effects were observed in the rostral HF for cage-reared hens. Regarding the relationship between hippocampal plasticity and the detour test outcome, birds making the detour had a higher multipolar cell density in the caudal HF than birds not making the detour. The distance walked during the open field test was positively associated to the multipolar cell density in the caudal HF. No effects were found on the bipolar cell density for both the detour test outcome and the distance walked during the open field test.

4.4 Paper IV

Paper IV investigated the long-term effects of environmental complexity during rearing on plumage quality and stress responsivity. The plumage quality of the hens on the head and neck, and back and rump was not affected by the rearing nor the adult

environment. However, contrary to our expectations, hens housed in enriched furnished cages during the production period had a poorer plumage quality on the belly than hens housed in standard cages, regardless of their rearing environment. Regarding stress responsivity, hens from the stress group showed a higher increase in plasma corticosterone levels than hens from the control group. The rearing environment had no effect on the hens' stress responsivity, but hens housed in enriched furnished cages had an overall lower level of plasma corticosterone than hens housed in standard cages.

5 Discussion

The aim of this thesis was to get a better knowledge of the effects of rearing environmental complexity and adult environmental enrichment on laying hens, with a focus on medium- and long-term effects. The results showed that the environment experienced by the hens during rearing or the production period affected them differently.

5.1 Effects of the rearing environment complexity

The effects of the rearing environment were studied in all papers, with **Paper I** investigating both medium- and long-term effects on fearfulness, while **Paper II** focused on the medium-term effects on cognition. **Paper III** and **IV** studied long-term effects on spatial cognition and hippocampal plasticity, and stress responsivity and plumage quality, respectively. Overall, the results showed that aviary-reared birds had better cognitive abilities than cage-reared hens. Contrary to predictions, no significant differences were observed between the rearing groups regarding fearfulness, stress responsivity and plumage quality. However, fearfulness was related to performances in cognitive testing, which was associated with the environmental complexity during rearing.

5.1.1 Effects on behaviour and plumage quality

Contrary to predictions, the early-life environment had little effect on fearfulness in laying hens (**Paper I**). Aviary-reared hens and cage-reared hens did not show significant differences in the distance walked in the open field at 33 nor at 61 weeks of age, nor on the latency to leave the start area. The latency to start exploring the arena and the distance walked are usually used as measures of fearfulness, with longer latencies and shorter distances walked reflecting higher fear levels (Forkman et al., 2007). The same results were observed in the novel object test, with hens from both rearing environments displaying not significantly different latencies to approach the novel object at 31 and 60 weeks of age. The results from both tests suggest the rearing environment complexity had little medium- and long-term effects on hens' fearfulness. A previous study using a similar rearing design showed that cage-reared hens were more fearful than aviary-reared hens when tested at 19-23 weeks of age, as shown by the higher amount of time spent close to a novel object or

human by aviary-reared birds (Brantsæter et al., 2016a; Brantsæter et al., 2016b). When the findings from this previous and the current work are seen together, they indicate that the rearing environment has short-term effects on fearfulness, but that the effects eventually fade over time, at least when housed in the adult environments used for the present thesis. It is supported by the fact that, when tested at 33 weeks of age, there was a trend for cage-reared hens to be slower to approach the novel object than aviary-reared hens (**Paper I**). This tendency disappeared when hens were tested at 61 weeks of age. It was argued that the differences in fearfulness between cage- and aviary-reared hens could be partly due to the greater amount of time spent by the farmers in contact with the hens in aviary systems compared to cage systems (Brantsæter, 2017). In our study, hens were all housed in furnished cages during the production period, which limited the human contact experienced by the hens once they were transported from the rearing farm. It could be that reduced fearfulness due to human contact is not hardwired, and exposure to humans and procedures in the henhouse need to be repeated to last over time.

However, as predicted, the rearing environment was important in the development of cognitive abilities and had long-lasting effects. Results from the holeboard test and habituation phase showed differences between hens from the two rearing groups (**Paper II**), and the same was observed with the results from the detour test (**Paper III**). During the habituation sessions prior to the holeboard test, cage-reared hens ate fewer mealworms than aviary-reared hens. Though this could be explained by differences in fear levels and lower exploration of the arena, results from the novel object and open field tests indicate that this is not the case. It could however be that exposure to a set of novel objects (rewarded cups) in an unfamiliar environment (holeboard arena) triggered a stronger fear response than being exposed solely to a novel object or an open field. During the testing phases, aviary-reared hens were overall faster to find the first bait than cage-reared hens and showed a better reference memory at the beginning of the cued phase. The reference memory is used as a measure of discrimination between baited and non-baited cups (van der Staay et al., 2012), which suggests aviary-reared hens might have associated faster the cues with the presence of rewards. Alternatively, this difference might be due to cage-reared hens showing a stronger neophobic reaction to the introduction of cues in the arena. Regarding (general) working memory ratios, hens from both rearing environments performed similarly in our study. This differs from what has been previously reported by Tahamtani et al. (2015), with aviary-reared hens showing higher working memory ratios than cage-reared hens. This difference might arise

from the slight modifications made to the holeboard set-up used in this thesis (discussed in section 5.5.2), or from the age of the birds. In this thesis, the focus was on the medium-term effects of the rearing environment complexity on cognition and birds were tested from 35 weeks of age, i.e. 17 weeks following transfer to the production farm. In Tahamtani et al. (2015), hens were tested at a younger age, starting two weeks after transfer to the production farm. In Skånberg et al. (2023), chicks reared in a more complex environment (more litter and perch types) were faster to perform a detour at 26-36 days of age than chicks housed in control housing. Similarly, Norman et al. (2019) found that chicks reared with elevated structures were faster to perform the detour than chicks from standard environments both at 2 and 4 weeks of age.

The results from the detour test reported in **Paper III** point in the same direction and suggest the effects of environmental complexity during rearing on the ability to solve a detour task are long-lasting. When tested at 62 and 64 weeks of age, aviary-reared birds were faster and more likely to perform a detour than cage-reared hens. The ability to perform a detour is usually used as a measure of spatial cognitive abilities, also in poultry (Regolin et al., 1995; Regolin & Rose, 1999). Our findings suggest that the environmental complexity experienced by the birds during the rearing phase can have long-lasting effects on their spatial cognitive abilities. Some effects of the rearing environment on spatial skills have previously been reported, with cage-reared hens displaying a lower use of the three-dimensional space than aviary-reared hens at the beginning of the production period (Brantsæter et al., 2016a). Similarly, hens reared with access to perches showed higher use of three-dimensional space later in life (Gunnarsson et al., 2000). The test used in Gunnarsson et al. (2000) requiring navigating vertically and jumping, the results might have been affected by the birds' physical strength. The tests used in this thesis only required the ability to walk on a flat surface, which put less pressure on the birds' physical abilities. Regardless, the results from this thesis go in the same direction and add to the body of studies investigating the effects of the early-life environment on cognition. It is however important to mention that the likeliness of performing the detour was associated with the distance walked during the open field test. This aspect is discussed in more detail in section 5.4.

The plumage quality assessment performed at 52 weeks did not reveal significant differences between hens from the two rearing environments (**Paper IV**). Overall, all hens had a good feather condition on their back and neck, scoring in the highest

category in over 70% of the cases. Damaged feathers or naked areas on the back are often associated with severe feather pecking, so the findings from this thesis suggest relatively low occurrences of severe feather pecking in hens from both rearing environments. Feather condition on the belly was poorer, but still comparable between both treatment groups. Conversely, other studies reported poorer feather conditions in birds reared in less complex environments, such as without early access to perches (Gunnarsson, 1999). Early and continuous access to litter during the rearing period also decreased the occurrence of feather pecking in young and adult hens (de Haas et al., 2014b; Tahamtani et al., 2016). It is worth mentioning that as aviary-reared hens did not display poorer plumage quality than cage-reared hens, it is unlikely they were experiencing high levels of frustration from being housed in a cage system during the production period. More detailed analysis of their behaviour, such as the expression of comfort behaviour, would nevertheless be needed to assess their actual experience.

5.1.2 Effects on physiology and hippocampal plasticity

In **Paper IV**, hens were tested between 52 and 54 weeks of age in a restraint stress test. Results showed a stronger increase in plasma corticosterone concentration for hens exposed to the restraint than control hens, validating the use of the restraint to induce a stress response. Hens from both rearing environments had similar overall levels of plasma corticosterone, implying the environmental complexity during rearing had no effects on the hens' levels of stress hormone as adults. Similarly, Brantsæter et al. (2016a) found no differences in faecal corticosterone metabolites between aviary- and cage-reared hens at 19 and 23 weeks of age. Provision of enrichment increasing environmental complexity also had limited effects on pullets' plasma corticosterone concentration at 16 weeks of age (Campbell et al., 2020). It therefore seems that increasing environmental complexity during rearing does not necessarily affect plasma corticosterone concentration later in life. Similarly, Prinold and Widowski (unpublished, in Widowski and Torrey (2018)) report that aviary- and cage-reared hens show no differences in faecal corticosterone following transfer to furnished cages.

Regarding effects on hippocampal plasticity, hens from both rearing environments had similar bipolar DCX+ cell densities in the rostral and the caudal HF at 65 weeks of age (**Paper III**). This result suggests that the rearing environment experienced by the hens had no long-term effects on cell proliferation and/or maturation in the two

subregions. There were however some effects of the rearing environment on the multipolar DCX+ cell density, as shown by the three-way interaction between the rearing and adult environment and the HF subregion. This effect is discussed in more detail in section 5.3.

5.2 Adult enrichment

Enriched environments are usually beneficial to improve animal welfare by stimulating cognitive abilities and decreasing fearfulness and stress responses. Results from this thesis support some of these effects, but not all. The enrichment used in the cages aimed at enabling and facilitating the expression of more behaviours by providing an additional dustbathing platform and a pompom to peck at. In addition, the structural complexity of the cage was increased by using curtains acting as visual barriers. The hens had to walk around them or push through them to navigate in the cage. As a way to slightly increase the structural complexity of the cages, the position of the dustbathing platform and the pompom was changed to the other side of the cage every other month.

5.2.1 Effects on behaviour and plumage quality

As predicted, the provision of environmental enrichment during the production period reduced the latency of the hens to approach a novel object in the home cage (**Paper I**). This effect was independent of the rearing environment and suggests that environmental enrichment can decrease neophobic reactions towards a novel object in laying hens. It is however important to consider that the hens tested in this study were all housed in furnished cages during the production period, which is relatively less stimulating than alternative housing systems. Enrichments provided in furnished cages might therefore have stronger effects than when provided in an aviary. For example, recent work showed that environmental enrichment had little effect on fearfulness in hens housed in aviaries (Tahamtani et al., 2022). The effects on fearfulness were however not consistent in the open field test, with hens housed in additionally enriched cages showing similar latencies to leave the start area and distance walked as hens in standard furnished cages. These discrepancies highlight the importance of taking several measures when assessing fearfulness. Some potential reasons behind these discrepancies are discussed in 5.5.3.

In contrast and contrary to predictions, the provision of environmental enrichment had no significant effects on the hens' ability to solve a detour task (**Paper III**). In a

wide range of species, the provision of environmental enrichment improved cognitive abilities (Bolhuis et al., 2013; Grimberg-Henrici et al., 2016; Hedges & Woon, 2011; Peña et al., 2009). In chicks, providing structural enrichment led to better cognitive abilities of the birds (Norman et al., 2019). Hens tested in the detour test were close to the end of the production period and aged over 60 weeks. It could be that at that age the effects of environmental enrichment were not as strong as in still developing chicks. Alternatively, it could be that the opportunity for the hens to navigate between the two parts of the cage represented a relevant source of complexity to solve the detour task. Providing curtains in the additionally enriched furnished cages might therefore not have been a significant increase in structural complexity compared to the standard furnished cages.

Regarding the plumage quality, birds housed in enriched furnished cages showed a poorer feather condition on their belly than hens housed in standard furnished cages. Damage to feathers on the belly is usually associated with highly productive animals, but also with vent pecking (Savory, 1995). The latter is usually characterised by the presence of wounds and cannibalism (Pötzsch et al., 2001), as pecking is directed towards the mucous membrane of the cloaca (Savory, 1995). No wounds were observed on the belly of the hens while scoring, making it unlikely that the damage was due to vent pecking. In addition, no significant differences were found in plumage quality for the other two body parts (back/rump and neck), suggesting no differences in severe feather pecking between hens housed in additionally enriched furnished cages or not. It is possible that the substrate used as dustbathing material induced some damage to the feathers, or that the material used to build the dustbathing platform (wood) wore the feathers down. As no behavioural observations in the cage were performed, it is not possible to state the reason for these differences in plumage quality with certainty.

5.2.2 Effects on physiology and hippocampal plasticity

Results from the restraint stress were influenced by the provision of environmental enrichment. Hens housed in additionally enriched furnished cages had an overall lower plasma corticosterone concentration than hens housed in standard furnished cages, suggesting environmental enrichment can reduce the stress response when facing a challenge. This is similar to what Asher and Bateson (unpublished, in Bateson and Matheson (2007)) report, stating that housing starlings in enriched cages reduced the corticosterone levels compared to starlings housed in barren cages.

In general, the provision of environmental enrichment is known to promote adult hippocampal neurogenesis in rodents (Kempermann et al., 1997; van Praag et al., 2000). Similarly, housing pigeons in enriched cages increases the density of DCX+ cells in the hippocampus (Melleu et al., 2016). Results from the immunohistochemistry analysis performed in this thesis showed that providing enrichment in the cages did not affect the bipolar DCX+ cell density in the HF, meaning no effects on cell proliferation and/or maturation. However, as previously mentioned, there was a three-way interaction between the rearing environment, provision of adult enrichment and HF subregion for the DCX+ multipolar cell density. Differences in the multipolar cell density reflect differences in the survival of the DCX+ cells. In rodents, the provision of environmental enrichment particularly affects the cell survival in the hippocampus (Kempermann et al., 2002), which goes in the same direction as the effects observed on the multipolar DCX+ cell density of the hens. The interaction effects are discussed in the next section (section 5.3).

5.3 Interaction between early-life and adult environments

Adaptive developmental plasticity (ADP) theories presented in section 1.4 indicate that the early-life environment is important to shape individuals' development, and that effects on individuals' fitness might differ depending on the adult environment they experience. The informational ADP proposes that the rearing and adult environments should match to provide the individual an advantage in fitness, while the somatic state-based ADP suggests that the presence of some characteristics of the rearing environment will always provide an advantage to the individual's fitness. In the context of this thesis, these theories were applied to single outcome measures and welfare rather than fitness. Based on previous research, we expected birds reared in cages to have poorer welfare than aviary-reared hens, which would be translated by a higher fearfulness and stress responsivity, and lower cognitive abilities. Similarly, increased environmental complexity during the production period by providing environmental enrichment should promote individuals' welfare. If the informational ADP applies, we would expect that transferring cage-reared birds to additionally enriched furnished cages will decrease their welfare, while aviary-reared hens will have poorer welfare once transferred to standard furnished cages.

Overall, there were little interaction effects between the rearing environment and the adult environment. As previously discussed, the rearing environment had effects on the development of cognitive abilities, and the provision of enrichment during the

production period affected fear and stress related responses in adult hens. But these effects were independent of each other, and housing hens with additional enrichment did not affect their responses and performances in the tests differently based on their rearing environment.

The fact that aviary-reared birds performed better in cognitive tasks than cage-reared hens seems to support the somatic state-based ADP, with higher environmental complexity during early-life promoting spatial skills. However, it can be argued that when transferred to a cage system for the production phase, these higher cognitive abilities might not be adaptive. This could lead to frustration from being housed in a less stimulating environment and *in fine* to poor welfare. Despite this mismatch between the rearing and production environment, aviary-reared hens did not show poorer plumage quality than cage-reared hens in body parts associated with feather pecking, suggesting they might not experience a high degree of frustration. It is nevertheless important to mention that these ADP theories were first developed with fitness as an outcome measure, and applying them to single outcome measures (such as cognitive abilities or stress responsivity) might require some adjustments.

There was however an interaction effect regarding hippocampal plasticity, with the provision of environmental enrichment during the production period affecting the multipolar DCX+ cell density in the two HF subregions differently based on which environment the hens experienced as chicks. While provision of environmental enrichment increased the DCX+ cell density in the rostral HF for cage-reared hens, the effects of enrichment were observed in the caudal HF for aviary-reared hens. In birds, the rostral and caudal HF are thought to be homologous to the dorsal and ventral subregions of the mammalian hippocampus (Smulders, 2017). Studies on rodents showed that anxiety was associated with reduced DCX+ cell density in the ventral hippocampus (Anacker et al., 2018), while exposure to chronic stress decreased DCX+ cell density in the caudal but not the rostral HF in laying hens (Gualtieri et al., 2019). This suggests the provision of environmental enrichment to aviary-reared birds might have reduced their experience of chronic stress, as shown by the higher density of multipolar DCX+ cells in the caudal HF. In Gualtieri et al. (2017), housing mice in enriched cages increased DCX+ cell density in the dorsal hippocampus, which is similar to the effects observed in the rostral HF of cage-reared hens. As the rostral HF is believed to be involved in spatial cognition, it seems that the structural enrichment provided in the additionally enriched furnished cages stimulated hippocampal plasticity that could improve cognitive abilities in cage-reared chicks. This points in a

different direction than the results obtained on behavioural and physiological measures and suggests that the informational theory might apply in this specific case. Indeed, the mismatch induced by housing hens reared in an aviary in standard furnished cages during the production phase had a negative effect on the cell density in the caudal HF. Conversely, the mismatch induced by housing cage-reared hens in additionally enriched furnished cages did not negatively affect the level of hippocampal plasticity in the caudal HF, but on the contrary, it enhanced the cell density in the rostral HF. It could be that the valence of the direction of the match/mismatch affected the birds differently. Indeed, hens reared in the aviary went from a highly complex environment to a more basic and less stimulating cage system. On the other hand, cage-reared hens were housed in a similar (standard furnished cages) or more stimulating (additionally enriched furnished cages) environment than their rearing environment. A mismatch from a poor to a better environment might therefore not have negative effects if the degree of complexity does not prevent individuals from accessing essential resources, such as food, water, and nests.

5.4 Relationship between hippocampal plasticity, cognition, and fearfulness

As mentioned in section 5.1.1, while rearing conditions did not have medium- or long-term effects on fearfulness, variables from the open field test did significantly associate with outcomes from the detour test (**Paper III**), indicating a subtle influence of fearfulness on the cognitive test. The distance walked in an open field arena is usually used as a measure of the birds' fearfulness (Forkman et al., 2007), which suggests that hens not making the detour might be more fearful than the ones making it. This difference might also arise from the fact that birds walking more during the open field test might explore the arena more, leading to a better representation of the arena. This could facilitate solving a detour test. However, the results discussed in the previous section and effects on hippocampal plasticity seem to support the fearfulness hypothesis best. In addition, adding the distance walked to the open field arena in the analysis of the detour test decreased the significance of the rearing environment, suggesting the two share some information, despite results from the fear tests being inconclusive. This suggests that the rearing environment can affect both cognition and emotivity in subtle ways. This relationship between the distance walked in the open field arena and the outcome from the detour test could also be due to differences in coping style, with birds walking more displaying a more proactive behaviour.

Some studies have reported associations between hippocampal neurogenesis or plasticity and cognitive abilities in rodents and in birds (Anacker & Hen, 2017; LaDage et al., 2010; Patel et al., 1997). In **Paper III**, the ability to perform a detour was related to the density of multipolar DCX+ cells in the caudal hippocampus. Hens performing the detour had a higher multipolar DCX+ cell density in the caudal HF than hens not performing the detour. In birds, the caudal hippocampus is thought to be involved in the regulation of the stress response and is sensitive to emotional stimuli (Smulders, 2017) and chronic stress (Gualtieri et al., 2019). Similarly, lower adult neurogenesis in the ventral hippocampus is associated with higher anxiety in mice (Anacker et al., 2018). The results from the detour test and hippocampal plasticity analysis could suggest that hens not performing the detour are more prone to anxiety than the ones performing it. This would support previous findings on rodents showing that exposure to chronic stress impairs cognitive abilities (Eiland & McEwen, 2012; Krugers et al., 1997). Alternatively, it could be that the gradient of specialisation of the avian HF is not as strict as it is thought, and the caudal HF might also be involved in processing spatial information.

5.5 Methodological considerations

5.5.1 Housing

During the rearing phase, cage-reared hens were kept inside one of the aviary rows to simulate cage rearing. This prevented the hens from navigating between the different tiers of the aviary and from accessing the floor of the house and litter. Though this reduced the environmental complexity, chicks still had access to perches within the aviary row, which is not necessary the case in all commercial cage-rearing system. Perch access could have represented a form of environmental enrichment and increased the complexity of the environment of the chicks, potentially leading to the development or improvement of three-dimensional navigation skills.

The different types of housing used during the production period also did not differ greatly. As detailed in the methods section, the hens were housed in groups of four in two commercial cages adjoined by an opening. The commercial cages used are designed to house up to ten hens, which is over four times the number of birds in our design. The hens had potentially an easier access to the different resources (nest boxes, perches, dustbathing platforms) than in a standard commercial setup, which could decrease social competition and the experience of social stress (EFSA Panel on

Animal Health and Animal Welfare, 2023). This could also be one of the reason the environmental enrichment had little effects on the plumage quality of the birds.
In addition, the difference in terms of complexity between the two types of cages used during the production period is not extreme. As the hens were housed in small groups and had an easier access to the enrichment of the furnished cages, the provision of environmental enrichment might not have been a great determinant in structural complexity. It is however noteworthy to observe that, despite the low degree of difference between the two cage types, some effects were observed on fearfulness and stress responsivity.

5.5.2 Cognitive testing

When performing tests to assess cognition, it is important to consider potential confounding factors. For example, results in cognitive tests might be affected by underlying levels of fearfulness, or differences in the physical abilities of individuals. Typically, some individuals could perform poorly in a three-dimensional task if their rearing environment did not allow them to develop the physical strength to jump, fly or walk in the arena. The cognitive tests used in **Paper II and III** did not require more than the ability to walk, which can be achieved with a standard physical condition. It therefore seems unlikely that differences in physical abilities could have affected the results, especially considering the birds were previously tested in an open field test (**Paper I**). The results from the open field test indicated no differences in the distance walked in the arena between the groups, suggesting that hens from all groups possessed the physical prerequisites for cognitive testing in the detour and holeboard test. However, even with good physical condition, results from cognitive tests can be affected by fearfulness. It has previously been shown that complex rearing environments can decrease fearfulness in hens (Brantsæter et al., 2016b; Nazar et al., 2022). However, results from **Paper I** showed no differences between cage- and aviary-reared hens in both the novel object and open field tests, suggesting results from the cognitive test should not have been strongly affected by fearfulness.

As already briefly mentioned, the selection process prior the holeboard test (**Paper II**) might have affected the results. Though necessary to ensure hens will engage with the task during the testing phase, picking the best performing hen for the test led to losing some of the variation existing within each group. As seen with the number of worms eaten during the habituation phase, cage-reared hens ate overall fewer worms than the aviary-reared hens. It is impossible to state for sure whether these

differences arose from a higher fear level or from lower cognitive abilities. However, as previously discussed, results from the fear tests reported in **Paper I** suggest few effects of the rearing environment on fearfulness. This might favour the hypothesis of differences in cognitive abilities. Another factor that could affect the results from the holeboard test is the fact that checking the cups is not costly for the hen. Hens can easily check the content of the cups when going from one cup to another, leading to lower memory ratios. Covering the top of the cup to make it harder for the hen to check its content might be a way to get clearer results. Differences in the memory ratios reported in **Paper II** and previous studies using the holeboard can be due to the slightly different design used. In Nordquist et al. (2011) and Tahamtani et al. (2015) a 3 x 3 matrix of cups was used. Due to space constraints to build the arena inside the hen house, a 4 x 2 matrix of cups was used in the holeboard test performed in this thesis, which might be slightly easier to navigate. Randomising the start position of the trial to prevent the learning of a fixed route to the rewarded cup was, on the other hand, increasing the complexity of the task.

5.5.3 Open field vs novel object test

In **Paper I**, hens were tested in a novel object and an open field test to assess their fearfulness. As previously discussed, the rearing environment did not significantly influence the outcome of both tests, but the provision of enrichment had some effects. While no effects were observed during the open field test for both the latency to leave the start area and the distance walked in the arena, the provision of enrichment did decrease the latency to approach a novel object. Several factors could explain the discrepancies between the two tests.

First, the social environment during the test could have affected the results. The novel object test was performed directly in the home cage, whereas the open field test was performed in a novel arena. These two tests measured fearfulness at the group and at the individual level, respectively. By taking measures at the group level, the results of the test were not affected by potential effects of social isolation. However, since only the latency for the first bird to enter the cage-half with the novel object was recorded, results from the test only reflect the fearfulness of the boldest bird in the cage. Taking individual measures of latency to approach the novel object might therefore show different results.

In addition, the handling and transportation from the home cage to the open field arena might have affected the results from the test. As seen in the results of the stress test performed in **Paper IV**, the simple handling of the hens induces a stress response, as shown by the increase in plasma corticosterone in hens from the control group. The handling and transportation of the birds could therefore have triggered a stress response, potentially leading to higher fearfulness in both groups, making them more similar. Supporting this, chicks which were exposed to acute stress before a tonic immobility test showed higher duration of tonic immobility than control chicks (Marin et al., 2001).

5.6 Implications

Results from this thesis support that environmental complexity during rearing was beneficial to improve cognitive abilities up to the end of the production period. Despite having little effect on the fearfulness and stress responsivity, rearing hens in a complex aviary system compared to a simpler cage system might be a way to promote the hens' ability to cope with challenges and solve them, especially when housed in alternative systems. On the other hand, fearfulness and stress responsivity were more affected by the current environment and the increase in environmental complexity through the provision of environmental enrichment than the rearing environment. This suggests that providing additional enrichment during the laying period could improve the welfare of laying hens. It is important to consider that the hens used in this study were all housed in furnished cages for the production period, and the enrichment might not have as strong an effect on hens housed in alternative housing systems. This study however has implications for basic research and adds to the body of knowledge on the effects of early and adult environments on the traits of individuals. This thesis also reported results linking the hippocampal plasticity and individuals' behavioural traits, which has not been extensively studied before. Despite the effects of the rearing environment on fearfulness and stress responsivity fading over time, it does not mean no attention should be given to the rearing period. Long-term effects were reported on cognitive abilities and hippocampal plasticity, and the birds might still experience poor welfare during the first few months following transfer to the production farm.

6 Conclusion

The main aims of this thesis were to study the medium- and long-term effects of the rearing environment complexity and adult enrichment on laying hen characteristics. This thesis particularly focused on the effects on fearfulness, stress responsivity, cognition, and hippocampal plasticity.

The results from this thesis showed that both the environmental complexity during rearing and the production period had effects on the hens' characteristics. The rearing environment has few medium- and long-term effects on fearfulness (**Paper I**) and stress responsivity (**Paper IV**). However, it had long-lasting effects on spatial cognition, as shown by the results of the detour test (**Paper III**). Results from the holeboard test and the habituation phase (**Paper II**) also suggest some effects of the environmental complexity during rearing on the hens' cognition. Put in perspective with ADP theories, these results support the somatic state-based ADP, with complex environments promoting enhanced cognitive abilities throughout life. Conversely, the provision of environmental enrichment had little effect on the hens' spatial cognition in the detour test, but reduced fearfulness toward a novel object and decreased stress responsivity. Despite the lower plumage quality on the belly of hens housed in enriched furnished cages, the housing had little effect on plumage quality and, consequently, severe feather pecking.

It therefore seems the environmental complexity during early life has long lasting effects on spatial cognition, while the current environment seems to be more important to influence fearfulness and stress responsivity.

7 Identified gaps for future study

The results of this thesis shed light on some of the effects of environmental complexity and enrichment on laying hens. Using hippocampal plasticity as a measure of the hens' cumulative experience gave insights which were not reflected in the behavioural or physiological measures studied. It would therefore be interesting to investigate further the effects of environmental complexity on hippocampal plasticity, by taking measures at different life stages. Relating those measures of plasticity to performance in cognitive testing and fearfulness assessment could increase our knowledge of how these characteristics interact and can affect each other. As mentioned in the introduction, experience of chronic stress can affect the expression of GR and MR in the HF, which could end in dysregulation of the HPA-axis. Studying the hippocampal GR and MR densities in hens from different environments would therefore be relevant, and additional material collected throughout this project could serve this purpose.

In addition, this thesis only studied one breed of hen and focused on a white strain. There are some differences in behaviour between brown and white strains, notably in terms of fearfulness (Rentsch et al., 2023). It would therefore be relevant to repeat some of the experiments to see if the effects are consistent across different breeds and strains. Analysing the behaviour of the individuals in their home pen would also be a good way to get a better assessment of the hens' welfare.

Furthermore, as discussed in the previous section, the two different environments used during the production period were rather similar, and it would be interesting to compare the performances of hens housed in an actual aviary versus a cage system, as these two environments would present a bigger gap in terms of environmental complexity. Including production system that provide an outdoor access could also be relevant, as more and more hens are housed in these alternative systems.

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Papers I - IV

Paper I

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Early life environment and adult enrichment: Effects on fearfulness in laying hens

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ABSTRACT

The environmental complexity, both during early and adult life, contributes to shaping individuals' fearfulness. The present study aimed at testing whether hens reared in an aviary were less fearful than hens reared in cages, and whether provision of additional enrichment during the laying phase could reduce fearfulness. We used White Leghorn laying hens (N = 384) reared in cages (N = 192) or in an aviary (N = 192) and then housed in furnished cages from 18 weeks of age, with or without the provision of additional enrichment. We tested naïve hens at 31 and 60 weeks of age in a novel object test and at 33 and 61 weeks of age in an open field test. Cage-reared hens had a latency to approach the novel object comparable to the one of aviary-reared hens when tested at 31 weeks of age ($F_{1, 17} = 2.71$; $p = 0.12$). At 60 weeks of age, birds housed in additionally enriched furnished cages were significantly faster to approach a novel object than birds housed in standard furnished cages for both rearing conditions ($F_{1, 61} = 19.02$; $p < 0.01$). Hens reared in cages walked distances comparable to aviary-reared hens in the open field arena at 33 and 61 weeks of age ($t = -0.33$; $p = 0.75$ and X^2 (1, N = 123) = 0.02; $p = 0.89$, respectively), and the provision of additional enrichment during the laying phase did not increase that distance (X^2 (1, N = 123) = 2.01; $p = 0.16$). We also did not observe any differences in the latency to start moving in the arena ($p > 0.05$). These results suggest that the environmental complexity during rearing had no medium- and long-term effects on fearfulness measured in the open field and novel object test. However, additional environmental enrichment during the laying phase had a stronger influence, reducing fearfulness towards novelty. This study suggests that environmental enrichment during adulthood can have positive effects on laying hens' fearfulness.

1. Introduction

Fearfulness can be defined as the individual 's predisposition to be easily frightened (Boissy, 1995; Jones et al., 1996). This trait is important to protect the animal from danger but can decrease welfare if responses to fear-inducing stimuli are disproportionate (Mills and Faure, 1990; Boissy, 1995; Jones et al., 1996). In farm animals in general, increased fearfulness is known to lead to difficulty in handling the animals and loss of productivity (Boissy and Erhard, 2014). In laying hens, increased fearfulness can lead to feather pecking (de Haas et al., 2014), smothering (Gilani et al., 2012) and to a higher risk of keel bone fracture (Harlander-Matauschek et al., 2015). In addition, fear is also associated with negative emotional states, which can in turn affect animal welfare negatively (Boissy, 1995).

The environment during early life contributes to preparing the individual to its future life (Bateson et al., 2014). During that time, the brain is very plastic and neuronal circuits are shaped to adapt to the current environment. This can have long lasting effects on neurophysiology and behaviour (Di Segni et al., 2018), notably with regards to fearfulness and response to novelty (Caldji et al., 2000; Pryce et al., 2005). For example, a more complex environment during early life has been shown to decrease individuals' fearfulness in rodents (Peña et al., 2009), pigs (Beattie et al., 2000) and broiler chickens (Tahamtani et al., 2018).

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In laying hens, the production system makes a clear division between early and adult life. Hens are normally reared in a rearing farm before being transferred to the laying facilities before the onset of lay at around 16–18 weeks of age. This life stage division makes laying hens well suited for research on how early and adult experience shape the behaviour and physiology of the individual. In the EU, battery cages are prohibited, and the industry is moving towards cage-free systems. Worldwide, however, cage housing is still prevalent, especially in the pullet phase (Schuck-Paim and Alonso, 2021). These two rearing environments are interesting as they present two distinct levels of environmental complexity. Individuals reared in cages grow in a relatively poor environment, with few opportunities to express natural behaviours and develop cognitive abilities. The aviary rearing system offers more opportunities to the individual with, among other things, the option to navigate in three-dimensional space and the option to perform more locomotor behaviours such as stretching. The difference in rearing systems can lead to individuals with different traits and behavioural phenotypes (Tahamtani et al., 2014, 2015; Brantsæter et al., 2016a).

Most studies have looked at short-term effects of rearing on adult behaviour, but the effects of early life environment, probably due to effect on the developing nervous system, could potentially have long lasting impact on the behaviour. Although early-life experiences are normally thought to have crucial impact on behavioural development, later-life environment may modulate these effects (Nicol et al., 2001). For example, the provision of environmental enrichment during the peripubertal period or adulthood has been shown to reduce anxiety in rats (Francis et al., 2002; Koe et al., 2016). While the understanding of later-life environment modulating the effects of early-life experience is of basic interest, it also has implication for the laying hen industry as exaggerated fearfulness responses can cause damage to the birds both in cage and free-range systems (Jones, 1996). We therefore aimed to explore both medium- and long-term effects of the rearing environment, and the effect of environmental enrichment provision during the laying phase, on fearfulness. We compared behaviours of individuals reared in cages or in an aviary and then transferred to furnished cages, with or without the provision of additional enrichment. Data was collected within a few months after birds were transferred to furnished cages at the production farm and again after several months of housing in additionally enriched or standard Victorsson T10 furnished cages. Because aviaries represent a more complex and stimulating environment, we predicted that hens reared in an aviary would be less fearful than hens reared in cages. We also predicted that enriched housing during the laying phase would partially compensate for the effects of the rearing environment so that birds reared in cages but provided with enrichment as adults would be less fearful than birds reared in cages and housed without enrichment.

2. Material and methods

2.1. Animals, rearing and housing

2.1.1. Rearing

This study was conducted using 384 Lohmann White Leghorn hens. The birds were either reared in an aviary (N = 192) or in cages (N = 192) until transport to the experimental farm at 18 weeks of age. All birds were reared in one single room measuring 15 m x 72 m at a commercial hatchery (Steinsland & Co.). The room contained 38,000 birds housed in raised NATURA Primus 16 system (Big Dutchman, www.bigdutchman.com, see Fig. 1). Cages measuring 12 m × 0.8 m × 0.6 m (length × height × width) were stacked in three tiers. Each tier contained a feed line and nipple drinkers. After hatching, birds were placed in the first and second tier of the system. The front of all cages was closed, and the floor of the cages was lined with paper until 4 weeks of age. For birds in the cage-reared condition, the front of the cage remained closed during the whole rearing period to simulate cage rearing. The cage was located in the second tier of one of the aviary rows and contained 250 birds. In the aviary-reared condition, the front of the cages in the first and second tier was opened from five weeks of age. Birds could move freely throughout the whole room by navigating through, over, or under the aviary tiers. Wood shavings were used as litter material on the floor of the room, and perches were available in the aviary rows over the water line and the feed line. Once the front of the cage was open, birds had access to perches on the front of the cage. Additional perches were also extended from the cage front at seven weeks of age (see Fig. 1).

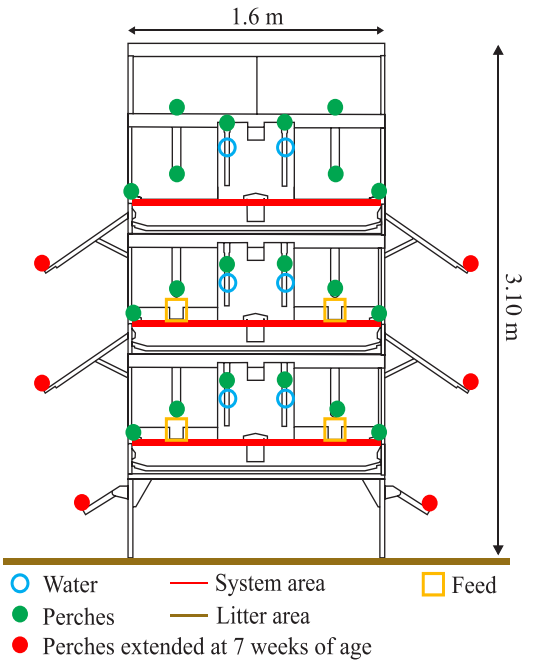

Fig. 1. Schema of Natura Primus 1600 viewed from the end of the row showing feed lines, water lines, and perches (based on the Big Dutchman leaflet).

From 5 weeks of age, the density was 26 birds/m^2 for the cage-reared birds and 29 birds/m^2 for the aviary-reared birds. All birds were exposed to the same lighting and feeding schedule. Temperature started at 34 °C and was gradually decreased to 19 °C at 16 weeks of age. Birds were exposed to 24 h of light for the first day, followed by a continuous 4:2 light/dark cycle during the first week as recommended by the Lohman LSL management guide. The light schedule was then switched to 16:8 light/dark at two weeks of age and gradually decreased to 9:15 light/dark by five weeks of age. Gradual transitions from dark to light and from light to dark were used. Each transition took 20 min. All birds received vaccination against coccidosis and Marek's disease.

At 18 weeks of age, 192 birds were randomly selected from the aviary (aviary-reared birds) and 192 birds were randomly selected from the tier which was kept closed (cage-reared birds).

2.1.2. Adult housing at the experimental farm

2.1.2.1. Description of the experimental facility. The henhouse contained 2808 cages organised in 12 rows. Each row contained 6 tiers, with a walkway between the 3rd and 4th tier, thus forming two floors in the building. The cages used for housing experimental birds were all situated on the third tier of the second floor, i.e. in the top tier. The four birds sharing a cage came from the same rearing treatment. The distribution of treatments in cages was balanced so that cages with birds reared in the aviary were always next to cages with birds reared in cages.

2.1.2.2. Type of housing during lay. After the arrival of the birds at the experimental farm at 18 weeks of age, they were housed in social groups of four in two Victorsson T10 cages that were adjoined by an opening measuring 15 cm × 18 cm. Each pair of cages containing four birds is hereafter referred to as a cage. Each cage measured 240 cm × 83 cm × 63 cm (width × height × depth). Standard control cages (n = 64) were furnished with two nest boxes, four perches and two metal dustbathing trays on the roof of each nest box (Fig. 2). Additionally enriched cages (n = 32) were the same as standard control cages with the addition of an extra dustbathing tray for stimulating foraging and dustbathing, a hemp pompon to peck at, and polyethylene tarp curtains and sheets to increase structural complexity. The latter were hung under one of the perches of the cage. In addition to this, a low-density polyethylene (LDPE) sheet was hung on the upper edge of each opening between the two cage-halves. Birds could therefore not see past these barriers and either had to move under or around them or push them out of the way to move past them. The extra dustbathing tray (55 cm × 60 cm width × depth with a 2 cm high frame to keep dustbathing material from falling off the tray) was placed on the perches in one half of the cage and refilled weekly with a mixture of feed crumbles and dustbathing pellets made of pelleted wheat husks. The pompon was attached to the cage front above the dustbathing platform so that it hung at the upper half of the cage wall. To slightly increase environmental variability, the position of the dustbathing platform and the pompon was switched to the opposite side of the cage every two months starting when the hens were 42 weeks old.

All birds were exposed to the same lighting and feeding schedule during their time at the farm. From the age of 18 weeks, they were kept under a 13:11 light/dark cycle and a temperature of 21.1 ± 1.6 °C without exposure to additional daylight from the outside. Gradual transitions from dark to light and from light to dark were used. Each transition took 15 min. Food and water were provided ad libitum via a food chain running in front of the cages and a water line with nipple drinkers along the back of the cages. For identification purposes, each bird was individually marked by means of a black or white plastic zip-tie around its left or right leg.

2.1.2.3. Experimental design. In total, 128 birds from each rearing environment were housed in standard furnished cages and 64 birds from each rearing environment were housed in additionally enriched furnished cages (see Fig. 3). The birds reared in cages or in an aviary and housed in standard furnished cages will be referred to as cage standard (cS) and aviary standard (aS). The birds housed in additionally enriched furnished cages will be referred as cage enriched (cE) and aviary enriched (aE). Half of the cS and aS birds (n = 16 cages/treatment groups, Fig. 3) were tested to study the medium-term effects of the rearing environment between 31 and 34 weeks of age. As part of another experiment, one bird per cage was removed at 24 weeks of age. The birds used to study the medium-term effects of the rearing environment were thus housed in groups of three from that age on. The other half of the cS and aS birds (n = 16 cages/treatment groups, Fig. 3) were tested along with the cE and aE birds (n = 16 cages/treatment groups, Fig. 3) to study the interaction effect of the rearing environment with the laying environment between 60 and 61 weeks of age.

2.2. Novel object test

A first novel object test was performed on hens housed in standard furnished cages at 31 weeks of age. The test was performed in the home cage of the birds. Birds in sixteen cages from each rearing condition were tested. At the beginning of the test, all hens from the same cage were gently moved to one half of the cage. A small eggcup glued onto a plywood square (19 cm × 19 cm) was baited with 3 mealworms (*Tenebrio molitor*; (Invertapro, Voss, Norway); known as a palatable food reward for hens; Moe et al., 2009) and placed in the empty part of the cage. The cup was placed in the middle of the cage-half used for testing and was visible from the other cage-half containing the birds. The food reward was visible once the hen entered the cage half containing the cup. The experimenter moved as far away from the cup as possible (~1.5 m) and started scoring. The latency to the first peck at the cup and total number of pecks at the cup were recorded. Considering the low occurrence of pecking behaviour observed, the latency to enter the cage-half with the cup (both legs had crossed the door between the two parts of the cage) was added to the list of variables recorded for nine cages of cage-reared birds and ten cages of aviary-reared birds. These variables were recorded at cage level, and the identity of the hen entering the cage-half with the novel object and being the first to peck

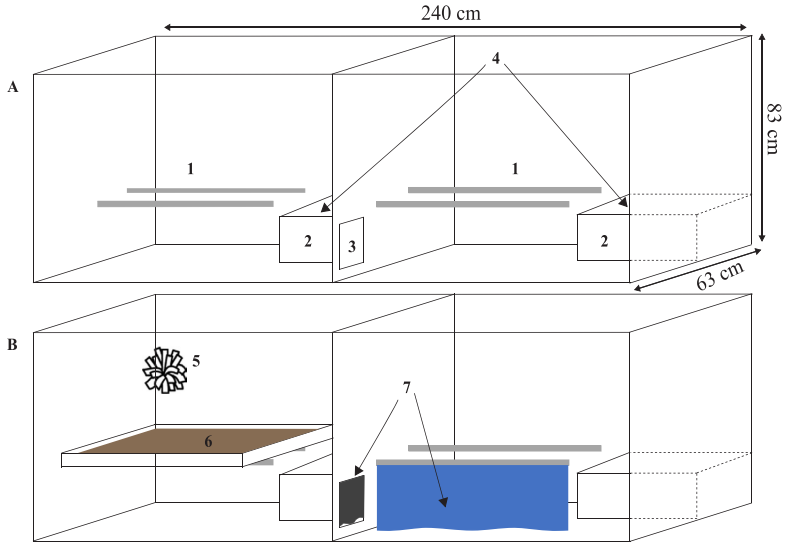

Fig. 2. Schemas of a standard Victorsson T10 furnished cage (A) and an additionally enriched Victorsson T10 furnished cage (B), three-quarter front view, showing (**1**) the perches, (**2**) the nest boxes, (**3**) the opening between the two parts of the cage, (**4**) the dustbathing trays, (**5**) the hemp pompom, (**6**) the additional dustbathing tray and (**7**) the curtains. The features **1**–**4** were also accessible in the additionally enriched cages.

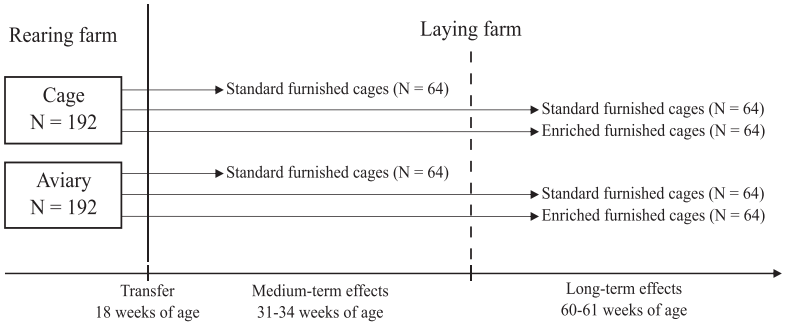

Fig. 3. Distribution of individuals in the different types of housing. A subset of bird was tested at 30–33 weeks of age and the other part at 60–61 weeks of age.

was not used in further analysis. The test was stopped after 5 min and the cup removed from the cage.

The novel object test was also performed on a different group of hens housed in standard furnished cages and additionally enriched furnished cages at 60 weeks of age. Birds from sixteen cages from each treatment group (aE, aS, cE, cS) were tested. The curtain between the two cage-halves was removed from the additionally enriched cages to ensure that the novel object was visible. The procedure followed was otherwise the same as the one described in the previous paragraph.

2.3. Open field test

The test was performed at 33–34 weeks of age on cage- or aviary-reared hens and at 61 weeks of age on different hens from the four treatment combinations (aE, aS, cE, cS). The two hens tested at each age were picked from the cages previously tested in the novel object test. The tests were conducted in two temporary arenas built in the hen house. In each arena, three of the walls were made of wood frames covered by a dark tarp, the fourth wall being the wall of the building in grey cement. The floor was made of particle board. Each arena measured 350 cm × 177 cm and walls were of 190 cm height. Lighting was provided by two lamps, one per arena, mounted on one of the walls of the arena. One camera (Axis m1124-e network camera, Noldus, The Netherlands) was mounted on the cement wall over each arena, at approximately 2.5 m of height, to allow the recording of the trials. All trials were recorded using the MediaRecorder system (Noldus Information Technology, Wageningen, The Netherlands).

At 33–34 weeks of age, 30 birds from each treatment group were tested. Two birds from the same cage were transported to their respective arenas and tested alone, one bird per arena, at the same time. The hen was placed in one of the corners of the arena and the test lasted for 10 min. At the end of the test, each arena was swept clean, and the hens were returned to their home pen. The corner of the arena used as the start point was the same for all tests.

For the second round of testing, naïve hens were 61 weeks of age (cS: N = 30; cE: N = 31; aS: N = 30; aE: N = 32). The hens were placed in a start box which was lifted by the experimenter from the outside of the arena to synchronise the start of the trials in the two arenas. The start box consisted of a grey plastic box measuring 40 cm × 30 cm × 20 cm (length × width × height) turned upside down. The procedure was otherwise the same as the one described for the first round of testing.

Videos were analysed using EthoVision X9 (Noldus Information Technology, Wageningen, The Netherlands). The latency to move and the total distance moved were recorded. The latency to move was defined as the central point of the hen's body crossing the line of the start area. The start area was a zone of approximately 40 cm × 40 cm in the corner of the arena where the hen was placed at the beginning of the test. The total distance moved was estimated by tracking movement of the central point of the hen at a rate of five samples per second. The sample point was set at the previous location until the distance moved was more than 5 cm to prevent an overestimation of the total distance moved. The track was also smoothened based on five samples before and after the sample point.

2.4. Data analysis

All statistical analysis were performed with R, version 4.0.3 (R Core Team, 2021). We used linear (mixed effects) models (L(M)Ms) fitted by restricted maximum likelihood estimates. Details on the structure of each model can be found below, under the subheadings of the different tests. P values were calculated by Wald chi-square and Wald F-test. All models were checked for assumptions (homogeneity of variances and normal distribution of residuals) and raw data were transformed to fit the assumptions when necessary. Interactions between predictors were first included in the models. When not significant, the interaction was removed, and the model was run again. In the case of one of the main effects being significant, within groups comparisons were performed by running the model on a subset of the dataset.

2.4.1. Novel object test

Due to a very low occurrence of pecking on the cup (13 cases over the 83 cages tested during the two tests), only the latency for the first bird to enter the cage-half containing the cup was analysed.

For each round of testing (age = 31 or 60 weeks), very few cages had the maximal cut-off latency of 300 s (two and one cage, respectively), so we used a LM in place of a survival analysis. The latency for the first bird to enter the cage-half with the novel object was used as the response variable (data were root transformed to meet the assumptions of the LM) and the type of rearing environment as a predictor. At 60 weeks, the provision of enrichment during laying was added in the model as a predictor on its own and in the interaction with the type of rearing. However, the interaction between the rearing and laying environments was not significant ($p > 0.05$) and was thus removed from the model. The whole cage was used as the statistical unit.

2.4.2. Open field test

The total distance moved and the latency to move were used as response variables. The data were root transformed to meet the assumptions of the LMM. The rearing environment was used as a predictor, and the cage was used as a random factor to account for the lack of independence between hens from the same cage. For the second round of testing (age = 61 weeks), the laying environment (additionally enriched or not) was added in the model as a predictor and in the interaction with the type of rearing. However, as for the novel object test, the interaction between the rearing and laying environments was not significant ($p > 0.05$) and was thus removed from the model.

The total distance moved from the first round of testing (hens aged of 33 weeks) did not meet the homogeneity of variances criterion. The

values for each cage were thus averaged, and a Welch t-test for non-homogeneous variances was used in place of the LMM.

2.5. Ethical statement

The animals used in this study were enroled in a larger project. An application for permission to perform the animal studies was submitted to and approved by the Norwegian Food Safety Authority (FOTS ID 22443). The experiments were performed in a farm approved as an experimental facility, and the experimental hens were housed in compliance with the Norwegian legislation regarding the use of animals in research (Forskrift om bruk av dyr i forsøk).

3. Results

3.1. Novel object test

At 31 weeks of age, there was no significant difference in the latency to enter the cage-half with the novel object between hens reared in cages or in an aviary ($F_{1,\ 17} = 2.71$; $p = 0.12$; cage-reared: 131 s ± 35 s; aviary-reared: 72 s ± 25 s, Fig. 4A).

At 60 weeks of age, hens housed in standard furnished cages were significantly slower to enter the cage-half with the novel object than hens housed in additionally enriched furnished cages ($F_{1,\ 61} = 19.02$; $p < 0.001$; standard: 81 s ± 14 s; enriched: 27 s ± 4 s, Fig. 4C). This difference was significant both within the cage-reared ($F_{1,\ 30} = 9.7705$; $p < 0.01$; cS: 90 s ± 23 s; cE: 26 s ± 6 s, Fig. 4C) and the aviary-reared ($F_{1,\ 30} = 9.6384$; $p < 0.01$; aS: 72 s ± 16 s; aE: 28 s ± 5 s, Fig. 4C) groups. The rearing environment had no significant effect on the latency to enter the cage ($F_{1,\ 61} = 0.09$; $p = 0.76$; cage-reared: 58 s ± 13 s; aviary-reared: 45 s ± 9 s, Fig. 4B).

3.2. Open field test

There was no significant difference in the distance moved between the different treatment groups at 33 weeks of age ($t = -0.33$; $p = 0.75$; cage-reared: 8.95 m ± 1.67 m; aviary-reared: 8.32 m ± 1 m, Fig. 5A). At 61 weeks of age neither the rearing environment (X^2 (1, N = 123) = 0.02; $p = 0.89$; cage-reared: 8.57 m ± 1.03 m; aviary-reared: 8.59 m ± 1.04 m, Fig. 5B) nor the adult environment (X^2 (1, N = 123) = 2.01; $p = 0.16$; standard: 7.13 m ± 0.84 m; enriched: 9.96 m ± 1.16 m, Fig. 5C) had an effect on the total distance walked in the arena.

Cage-reared hens and aviary-reared hens also did not significantly differ in their latency to start moving at 33 weeks of age (X^2 (1, N = 60) = 1.21; $p = 0.27$; cage-reared: 269 s ± 37 s; aviary-reared: 209 s ± 31 s, Fig. 6A) or at 61 weeks of age (X^2 (1, N = 123) = 2.27; $p = 0.13$; cage-reared: 304 s ± 26 s; aviary-reared: 251 s ± 26 s, Fig. 6B). The provision of enrichment had no significant effect at 61 weeks of age either (X^2 (1, N = 123) = 1.20; $p = 0.27$; standard: 297 s s ± 26 s; enriched: 259 s ± 26 s, Fig. 6C).

4. Discussion

The aim of the experiment discussed here was to get a better understanding of the effects of environmental complexity during rearing and the laying phase on laying hens' fearfulness. Because of the known positive effects of environmental complexity on the developing and adult individual (Beattie et al., 2000; Francis et al., 2002), we predicted that hens reared in an aviary would be less fearful than hens reared in cages. We also predicted that exposure to additional enrichment during the laying phase would partially compensate for the effects of the rearing environment. The results show that the effects were not consistent across the tests and that environmental complexity affects fear responses differently depending on the test method chosen.

Effects of early life environmental complexity have mostly been studied with focus on the short-term effects. In a previous study using a similar housing design, hens reared in an aviary spent more time in the zones close to the novel object than cage-reared hens five weeks after transfer to furnished cages (Brantsæter et al., 2016b). However, the duration of this rearing effect was not investigated. Contrary to Brantsæter et al. (2016b), we found no significant differences between cage- and aviary-reared hens in the latency to approach the novel object at 31 and 60 weeks of age. At 31 weeks of age, there was still a trend going in the same direction as the findings from Brantsæter et al. (2016b), with aviary-reared hens showing a shorter latency to approach the novel object. This trend was not present at 60 weeks of age, i.e., 42 weeks after transfer to the laying farm. This suggest that the effects of the complexity of the rearing environment fade over time, starting already after 13 weeks of transfer to the laying farm. A study by Hocking et al. (2001) showed that the birds avoided the novel object less as they aged. The lack of difference in the latency to approach the novel object between the cage-reared and aviary-reared hens could thus also be due to the age of the individuals, and not only to the effects of the rearing environment fading over time.

In a recent study, the current provision of environmental enrichment to hens housed in aviary systems had no effects on behaviour in the novel object test (Tahamtani et al., 2022). In contrast, our study showed that the provision of environmental enrichment during the laying phase significantly decreased the fearfulness of the hens when exposed to a novel object at 60 weeks of age. Hens housed in additionally enriched furnished cages were significantly faster than hens housed in standard furnished cages to approach the novel stimulus independently of the rearing condition. The inconsistency in results may be due to the fact that the hens used in our study were housed in furnished cages, which

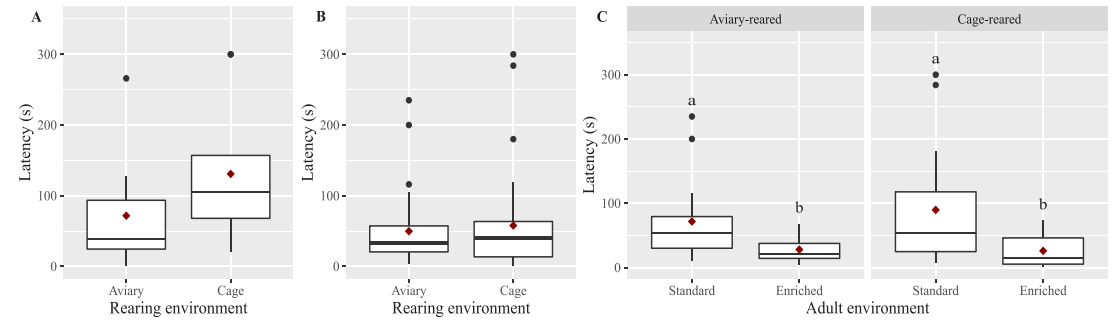

Fig. 4. Latency to enter the cage-half with the novel object at 31 weeks (A) and 60 weeks (B) of age for both rearing environments. The graph C shows the latency to enter the cage with the novel object at 61 weeks of age for each combination of rearing and adult environment. [a-b] Bars with no common letters differ significantly ($p < 0.05$).

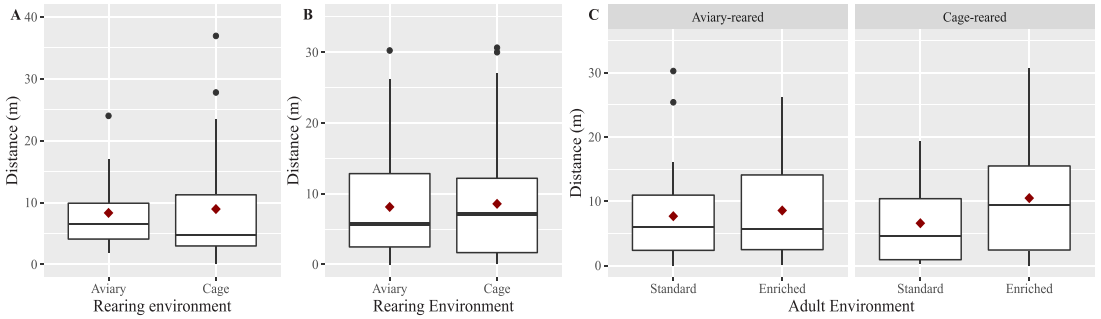

Fig. 5. Total distance moved during the open field in metres at 33 weeks (A) and 61 weeks (B) of age for the two rearing environments. The graph C shows the total distance moved during the test at 61 weeks of age for each combination of rearing and adult environment.

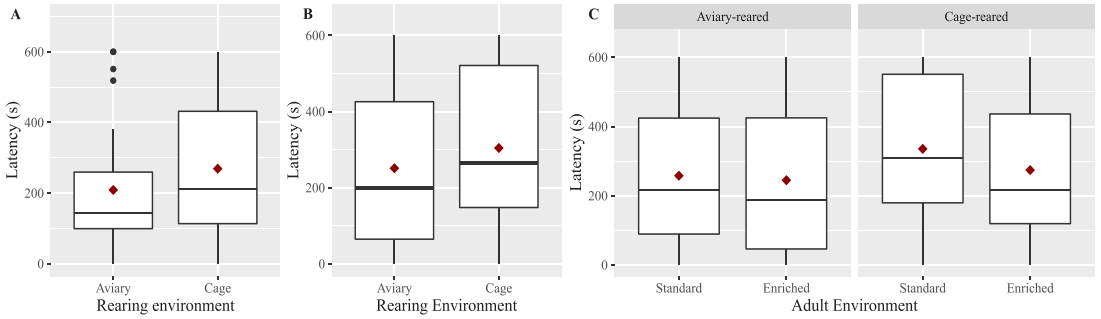

Fig. 6. Latency to start moving during the open field test in seconds at 33 weeks (A) and 61 weeks (B) of age for the two rearing environments. The graph C shows the total latency to start moving during the test at 61 weeks of age for each combination of rearing and adult environment.

represent a less complex environment than the aviary system studied by Tahamtani et al. (2022). The provision of environmental enrichment could thus be more beneficial for birds housed in cages and have a stronger impact as they face less stimulation than birds housed in more complex systems, such as aviaries.

The effects of environmental complexity and enrichment on the results of the open field test are not consistent with the results from the novel object test. Contrary to our predictions, we did not find any effects of the environmental complexity during rearing on the total distance moved in the open field test at 31 weeks of age. Hens reared in cages did not walk less in the open field than hens reared in an aviary, but showed the same level of exploratory behaviour. Increasing the environmental complexity during laying by providing additional environmental enrichment in the cage had no clear effects on the latency to move, nor on the total distance walked in the open field arena. The aviary-reared hens in our experiment came from an environment rich in stimulation but were transferred to furnished cages. Though the provision of enrichment increases the complexity of the cage and allows for the expression of more behaviours, it is still quite limited and represents a less complex environment than an aviary. That change from a more complex to a less complex environment could lead to frustration, as the birds are more restricted in their behaviours, and depression. For example, rats losing access to enrichment have been shown to express more depression-like behaviours than the control group (Morano et al., 2019; Smith et al., 2017). The results of our study could thus be affected by the loss of environmental complexity, which could lead to the aviary-reared hens not showing the expected higher degree of exploratory behaviour.

In laying hens, higher latency to move and reduced locomotion in an open field test are commonly used as indicators of higher fearfulness (Forkman et al., 2007). However, not all studies document the expected differences in fearfulness in this test. In Nordquist et al. (2011), chicks from two different lines were tested in a battery of tests to assess, among other things, fearfulness and anxiety. No differences were found between control and low mortality lines in the open field test, despite a difference in behaviour in the voluntary approach test. This is consistent with our results, failing to show any differences in the levels of fearfulness measured in the open field test despite clear differences between birds from the different treatment groups in the novel object test. Several factors might explain these differences.

First, birds must be transported from their home pen to the testing arena to be tested in the open field. The handling and transport, though gentle, can increase the stress levels of the birds and bias the measures taken during the open field test. Indeed, Fraisse and Cockrem (2006) and Littin and Cockrem (2001) studied plasma corticosterone concentration of hens in response to repeated handling. They both showed that repeated handling during 15 min was enough to elicit an increase in plasma corticosterone. This could lead to higher fearfulness. In Marin et al. (2001), chicks were exposed to acute stress before being tested in a tonic immobility test. Chicks subjected to acute stress before testing had a longer duration of tonic immobility than the control group, suggesting that acute stress induces higher underlying fear levels.

Another factor which could explain the difference in the levels of fearfulness measured between the open field and the novel object tests is the social environment. Indeed, those two tests as used in the current study measure fearfulness at the individual and group levels, respectively. Taking the measure at the cage level during the novel object test only reflects the latency to approach the novel object for the bravest bird of the cage. There is thus a loss of information on individual variability, and taking measures of more than one individual per cage might show

more differences between the treatment groups. In addition to that, chickens are social animals, and social isolation can increase stress levels. For example, socially isolated quail showed increased plasma levels of corticosterone even when isolated in a familiar environment (Hazard et al., 2008). Social isolation could increase the fear levels prior to testing to a level at which differences between the treatment groups are not noticeable anymore. The fear indicators measured in the open field are therefore the response to a novel environment, but also to social isolation. In contrast, the novel object test as used in the current study takes a group level measure of fearfulness since birds are tested directly in their home pen with familiar conspecifics. The output measure is thus the fear response to the novel object and is not affected by a change in the social environment. In commercial settings, individuals are rarely isolated and so measures at the group level might therefore be more relevant.

5. Conclusion

In conclusion, we found that rearing hens in different levels of environmental complexity had no medium- or long-term effects on the fearfulness measured in an open field and a novel object test. However, the provision of additional enrichment during the laying phase reduced fearfulness towards a novel object. These results suggest that environmental enrichment during adulthood can have positive effects on laying hens' fearfulness.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Paper II

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Effects of the rearing environment complexity on laying hens' spatial cognition: A holeboard test approach

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ABSTRACT

The rearing environment of layer chicks can differ greatly in degree of complexity. With the industry moving towards cage-free housing systems, greater demands are placed on the birds' cognitive abilities in order for them to find resources such as food, water and nest-boxes. Because early environmental complexity can influence cognition, we aimed at increasing our knowledge of how two different rearing environments affect the cognitive abilities of the hens. We habituated 64 hens to a spatial holeboard test, half of which were reared in cages and the other half in an aviary. Out of these 64 hens, 14 cage- and 14 aviary-reared White Leghorn hens were tested twice a day every workday in a holeboard test from 32 to 40 weeks of age. The test consisted of 4 consecutive phases, namely the uncued, cued, over-training and reversal phases, during which the hens had to find baits in a subset of cups in an arena. All cups were identical, so hens had to rely on spatial cues to find the baits which were always hidden in the same cups. During the cued phase, cues were added to the baited cups to give additional information to the hens. During the reversal phase, baits were hidden in a new subset of cups to study cognitive flexibility. The results show the birds were able to successfully complete the task. Aviary-reared hens had a higher reference memory score than cage-reared hens in the first block of the cued phase ($F_{1,26} = 4.21$, $p < 0.05$). Cage-reared hens also had a significantly higher latency to find the first bait than the aviary-reared hens for the uncued, cued and over-training phases ($F_{1,26} = 5.26$, $p < 0.03$; $F_{1,26} = 6.32$, $p < 0.02$; $F_{1,26} = 6.29$, $p < 0.02$). The same was observed for the transition between them (uncued-cued: $F_{1,26} = 6.19$, $p < 0.02$; cued-over-training: $F_{1,26} = 5.87$, $p < 0.03$). No significant treatment effects were found for the reversal phase. In conclusion, cage-reared hens were slower to find the first bait than aviary-reared hens and seemed to be more sensitive to changes in the environment, as shown by the differences during the transition between phases. Aviary-reared hens might therefore be better at adjusting to complex laying environments.

1. Introduction

In the egg industry, hens are usually kept in rearing farms before being transferred to the laying facilities. The type of environment experienced by the birds during the rearing period can differ greatly in degree of complexity. Indeed, in commercial production systems, chicks are usually reared in cage or aviary systems. The aviary system offers a much more complex environment, with among other things, the possibility for the chicks to dustbathe and to navigate in three dimensions by moving between the different tiers of the aviary. In both barren and furnished cage systems, chicks are confined to a smaller space where they can only access the tier they are housed in. After the rearing phase, pullets are transferred to laying facilities at 16–18 weeks of age where they are kept until 72–80 weeks of age. Since the ban on battery cages became effective in 2012 (Council of the European Union, 1999), birds in the EU are housed in furnished cages or alternative housing systems, such as barn, aviary or free-range. Partly due to welfare concerns from consumers and stakeholders, the industry is now moving towards cage-free housing systems. The shift from barren, less complex environments to environments presenting higher degrees of complexity demands more of the bird in terms of cognitive performance. Whether housed in a barn, aviary, or free-range systems, the birds must navigate their environment to find resources such as food, water, and nest boxes.

Rearing conditions are likely to affect cognitive abilities later in life.

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Early life is a critical period in development (Bateson et al., 2014; Di Segni et al., 2018): aversive experiences during early-life have long-lasting effects on the individual, including effects on cognitive abilities in mice and rats (Naninck et al., 2015; Alves et al., 2022). For laying hens, it means the environment experienced during rearing is an important factor influencing the development of the chicks (Janczak and Riber, 2015; Campbell et al., 2019). It has been shown that chicks which had no access to perches during early stages of life showed impaired spatial skills at the end of the rearing phase (Gunnarsson et al., 2000). It has also been shown that barren environments negatively affect spatial cognition in the short-term, up to seven weeks after transfer to the laying farm (Tahamtani et al., 2015). However, information is scarce on the longer-term effects of rearing in a barren environment.

Because early environmental complexity can influence cognition, we aimed at testing how two different rearing environments affect the cognitive abilities of the hens by using the spatial holeboard test (van der Staay et al., 2012). We focused on the medium-term effects of rearing on cognition and tested the hens between 32 and 40 weeks of age. The holeboard test is a task which has been used to assess different aspects of animal spatial cognition, such as learning and memory (van der Staay et al., 2012). It allows one to distinguish between working memory and reference memory. Working memory is a form of short-term memory, within a trial, whereas reference memory reflects long-term memory across trials. The spatial holeboard test has been used in several species, including in farm animals such as pigs (Arts et al., 2009; Roelofs et al., 2018), chickens (Nordquist et al., 2011; Ferreira et al., 2019) and more recently in calves (Lecorps et al., 2022). Compared to some other cognitive tests, such as the three-dimensional jump test, the holeboard test makes it possible to assess learning and cognitive abilities without the performances of the individuals being affected by their physical abilities.

To test the effect of early life environment on cognition, we reared hens either in a multi-tier aviary or in cages before transferring them to the laying farm at 18 weeks of age. The aviary representing a more complex environment, we expected hens reared in the aviary to show better cognitive abilities than hens reared in cages.

2. Material & methods

2.1. Animals, rearing and housing

2.1.1. Rearing

The hens used in this study (N = 64) were part of a larger project for which 384 non-beak trimmed White Leghorn hens were reared either in a cage (N = 192) or in an aviary (N = 192). They were then transported to an experimental farm at 18 weeks of age. The birds were reared at a commercial hatchery (Steinlands & co.) in one single room measuring 15 m x 72 m. The room contained 38000 birds housed in a raised NATURA Primus 16 system (Big Dutchman, www.bigdutchman.com, see Fig. 1). The system consisted of furnished cages measuring 12 m x 0.8 m x 0.6 m (length x height x width) stacked in three tiers. After hatching, chicks were placed on the first and second tier of the system. The mesh floor of the aviary rows was lined with paper until four weeks of age. Each aviary row was furnished with a feed line, nipple drinkers, and a perch above the water and feed lines. From 5 weeks of age, the front of the aviary rows was opened, and the birds could navigate between the different tiers and the floor of the house. They also had access to perches on the front of each tier of the aviary rows. The floor of the house was covered with wood shavings, and additional perches were extended from the front of each tier of the aviary rows at seven weeks of age (see Fig. 1). For one of the aviary rows, the front of one tier was kept closed during the whole rearing period. This enclosed space was located in the second tier of the aviary row and contained 250 birds. Thus, they had no access to the floor of the house or the other tiers of the aviary.

From 5 weeks of age, the density was 26 birds/m^2 for the cage-reared birds and 29 birds/m^2 for the aviary-reared birds. In the cage and aviary conditions, birds had access to 9.6 cm and 3.2 cm of perch space per bird, respectively. All birds were exposed to the same lighting and feeding schedule. Temperature started at 34 °C and was gradually decreased to 19 °C at 16 weeks of age. Birds were exposed to 24 h of light for the first day, followed by a continuous 4:2 light/dark cycle during the first week as recommended by the Lohman LSL management guide. The light schedule was then switched to 16:8 light/dark at two weeks of age and gradually decreased to 9:15 light/dark by 5 weeks of age. Gradual transitions from dark to light and from light to dark were used. Each transition took 20 min. All birds received vaccination against coccidiosis and Marek's disease.

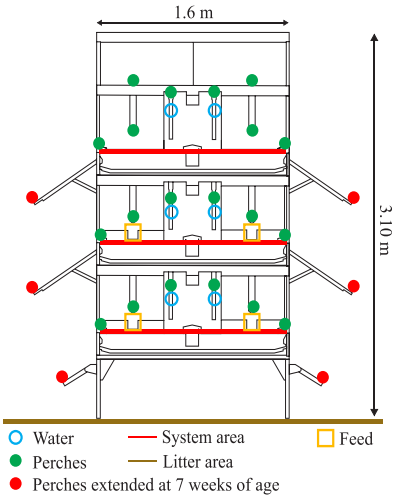

Fig. 1. Schematic representation of a raised Natura Primus 1600 viewed from the end of the row showing feed lines, water lines, and perches (based on the Big Dutchman leaflet).

At 18 weeks of age, 192 birds were randomly selected from the aviary (aviary-reared birds) and 192 birds were randomly selected from the tier which was kept closed (cage-reared birds).

2.1.2. Adult housing at the experimental farm

At 18 weeks of age, the birds were transported to the experimental farm. The henhouse contained 2808 cages organised in 12 rows, each row containing six tiers. A walkway between the 3rd and 4th tier formed the second floor in the henhouse. Experimental birds were all housed in the third tier of the second floor, i.e., the top tier. They were housed in social groups of four individuals in two Victorsson T10 furnished cages adjoined by an opening (15 cm × 18 cm). The opening between the two cages allowed the birds to move freely between the two cages of the cage-pair. Each pair of cages containing four birds is hereafter referred to as a cage. Each cage measured 240 cm × 83 cm x 63 cm (width x height x depth) and the four birds sharing a cage came from the same rearing treatment. Each cage was furnished with four perches (75 cm perch space / bird), two nest boxes (1500 cm^2 each) and a dustbathing platform on the roof of each nest box (750 cm^2 / bird, Fig. 2). The

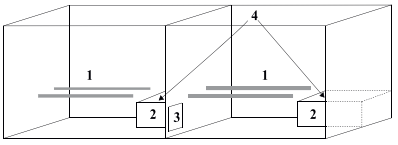

Fig. 2. Schematic representation of a furnished cage, three-quarter front view, showing (**1**) the perches, (**2**) the nest boxes, (**3**) the opening between the two parts of the cage and (**4**) the dustbathing trays.

treatments were distributed in the henhouse so that cages with birds reared in the aviary were next to cages with birds reared in cages. As part of another experiment, one bird per cage was removed at 24 weeks of age. The birds were thus housed in groups of three from that age on.

All birds were exposed to the same lighting and feeding schedule during their time at the farm. From the age of 18 weeks, they were kept under a 13:11 light/dark cycle and a temperature of 21.1 ± 1.6 °C without exposure to additional daylight from the outside. Gradual transitions from dark to light and from light to dark were used. Each transition took 15 min. Food and water were provided ad libitum via a food chain running in front of the cages and a water line with nipple drinkers along the back of the cages. For identification purposes, each bird was individually marked by means of a black or white plastic zip-tie around its left or right leg.

2.2. Holeboard test

From 32–40 weeks, birds were tested in a holeboard test modified from Tahamtani et al. (2015) and Nordquist et al. (2011). It consisted of a habituation phase, followed by a training and testing phase. A pilot study previously led by our group showed that 33% of the birds did not consume any mealworms after several days of habituation. We, therefore, habituated 64 birds from 32 cages (16 cages with aviary-reared birds and 16 cages with cage-reared birds) to identify hens not engaging with the task. We then selected a subset of birds (one per cage) for the testing phases (see details in the habituation section).

As part of the habituation phase, additional data was collected and the birds were tested in a novel object test and an open field test. More details on the methods and results are available in Dumontier et al. (2022).

2.2.1. Testing arena

Two temporary arenas were built in the henhouse to test the birds. Each arena measured 350 cm × 177 cm x 190 cm (length x width x height). Three of the walls were made of wood frames covered with dark green tarps, the fourth wall being the concrete wall of the henhouse. Each arena was illuminated with a lamp fixed on one of the walls. Eight circles of 50 cm diameters were drawn with a marker on the floor (particle boards) of each arena. Circles were spaced 20 cm apart and were distributed in a 2 × 4 matrix (Fig. 3). A small pink cup designed for holding a single egg was glued onto a 19 cm × 19 cm plywood plate and the plate was placed in the centre of each circle drawn on the floor of the test arena. In each arena, a grey plastic box turned upside down was used as a start-box (40 cm × 30 cm x 20 cm, length x width x height). The start-box was randomly positioned on one of the short walls for each trial session and kept in the same position for all hens tested during the same trial session. To start the test the start-boxes were lifted by the experimenter from the outside of the arena using a rope attached to a pulley system. In this way it was possible to synchronize the start of the test in the two separate arenas in which birds were tested at the same time.

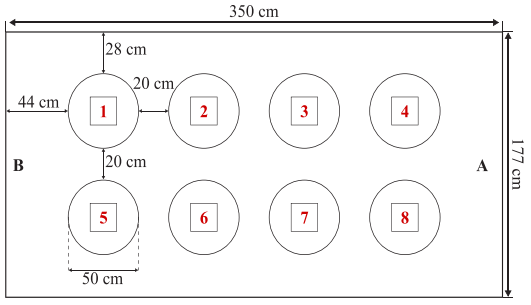

Fig. 3. Schematic representation of the holeboard arena, viewed from above. The letters A and B show the two possible positions of the start-box. Numbered squares represent the cups and plywood squares.

One camera (Axis m1124-e network camera, Noldus, The Netherlands) was mounted on the concrete wall of each arena at approximately 2.50 m to record the trials. All trials were recorded using the MediaRecorder system (Noldus Information Technology, Wageningen, The Netherlands).

2.2.2. Habituation phase

The habituation phase started when the hens were 32 weeks of age. They were habituated to the cups by three exposures per day for 5 days. During the first three days, they were presented with a small pink cup baited with three mealworms (known as a palatable food reward, Moe et al., 2009) directly in their cage. For the last two days of habituation, the cup was placed in the feed line. Each exposure to the baited cup lasted for 5 min, or until all mealworms were eaten.

Habituation to the arena was started when hens were 34 and 35 weeks of age. They were exposed daily for 5 days to the arena, each session lasting 5 min. During this habituation phase, all eight cups were baited with one mealworm to encourage exploration of the arena. For the first habituation session, two birds from the same cage were placed into the arena together to encourage them to explore. For the following sessions, they were exposed alone to the experimental setup. The number of mealworms eaten and the latency to eat the first mealworm were recorded for each habituation session. After habituation, the bird showing the best performance (number of mealworms eaten and latency to get the first reward) was selected for each cage. If no clear difference was observed between the two birds, one of them was randomly selected. Four cages (two with aviary-reared and two with cage-reared birds) were excluded because none of the hens consumed any mealworms. Thus, 14 cage-reared and 14 aviary-reared birds were included in the following holeboard test.

2.2.3. Training and testing phases

For all following phases, hens were trained/tested twice a day for 5 min except for the first day of the uncued phase where only one trial was performed. All tests were performed on workdays. Hens were always placed in the same arena and were returned to their cage between the two trials. The first cage tested was randomly chosen each day, and the order of testing (ascending or descending) was also randomly picked.

First, the birds were trained in an uncued phase for 12 days. During this phase, three cups out of eight were baited with one mealworm (see reward configuration section for more information). Then, the hens were trained in a cued configuration of reward for 4 days. The same configuration of baited cups as in the uncued phase was used, but the plywood squares under the baited cups were painted red (in place of the standard light brown colour) to give the hens additional cues. Following the cued phase, hens were trained for 4 days in an over-training phase. During this phase, baits were returned to their uncued form. Finally, hens were tested in a reversal phase for 5 days. During this phase, the hens were given a new configuration of uncued, baited cups.

2.2.4. Reward configuration

Across the 28 hens, 7 different configurations were used (4 hens per configuration, 2 cage- and 2 aviary-reared). Each configuration consisted of three mealworm-baited cups, and five empty cups. The configuration refers to the spatial position of the cup in the arena. The same configuration of baited cups was used during the uncued, cued and over-training phases. For the reversal phase, a new configuration of baited cups was randomly assigned to each bird.

2.2.5. Parameters recorded

For each trial, the latency to find the first bait (in seconds) and the trial duration (in seconds) were recorded. The trial duration was defined

as the time elapsed between the start of the trial and the visit to the last baited cup or the maximum cut-off of 300 s, whichever occurred first. The total number of cups visited, the total number of visits to the baited set of cups and the number of different cups visited were recorded. These parameters were used to calculate the following variables:

- **Working Memory Ratio (WM):** Number of rewarded visits divided by the total number of visits to the baited cups. Shows the capacity to avoid revisiting baited cups that have already been visited.
- **General Working Memory Ratio (GWM):** Number of different cups visited divided by the total number of visits. Shows the capacity to avoid revisiting cups that have already been visited.
- **Reference Memory Ratio (RM):** Total number of visits to the baited cups divided by the total number of visits to all cups. Reflects the ability to discriminate between baited and unbaited cups.

WM and GWM are measures of short-term memory and are trial dependant, whereas RM gives a measure of long-term memory and is not trial dependant. For all memory ratios, scores close to 1 indicate good performances and scores close to 0 indicate poor performances in the holeboard test.

2.3. Data analysis

All statistical analyses were performed with R, version 4.2.1 (R Core Team, 2022). For the habituation phase (N = 64 hens), we used generalised linear mixed effects models on the number of worms eaten by each hen, using the package *glmmTMB* (Brooks et al., 2017). As the hens were exposed to the arena in pairs during the first day of habituation, it was excluded from the analysis. The rearing environment, the habituation day and whether the bird was selected for the holeboard test were used as categorical predictors. Two-way interaction between the predictors were also included. The Individual ID nested within the Cage ID was used as a random factor to account for repeated measures across days and lack of independence between birds from a same cage. The same model was also run separately on birds which were selected (N = 28) or not selected (N = 36) for the holeboard test to see any difference between rearing environments. P-values were calculated by Wald chi-square tests and models were checked for overdispersion and homogeneity of variances.

For the holeboard test (N = 28 hens), we used linear mixed effects models (LMMs) fitted by restricted maximum likelihood estimates, using the package *nlme* (Pinheiro et al., 2021). Trials during which the hen dustbathed before completing the task were excluded from the analysis (24 trials over the 1372 performed in total). In addition, 8 trials were excluded due to recording or baiting issues. The response variables were averaged across blocks of two consecutive testing days for the analysis. As the reversal phase lasted 5 days, the first two and last two days were averaged but only data from the third day was used to calculate the average for day 3. The rearing environment, trial blocks, and the two-way interaction were used as fixed effects. The interaction was removed when it was not significant, and the model was rerun after it was removed. Each phase was analysed separately. The bird ID was used as a random effect in the model to account for repeated measures. To study the effects of changes in the arena (addition/removal of cues, change in the baits configuration), the transition between each phase was also analysed. The same model as previously described for the different test phases was run on a subset of data containing only the last and first blocks of two consecutive phases. P values were calculated by F-tests. All models were checked for their conformation to the assumptions of parametric statistical models (homogeneity of variances and normal distribution of residuals). Time variables (latency to find the first bait and trial duration) were log transformed to make them fit these assumptions. The RM for the transition between the Uncued and Cued phase did not fulfil the assumptions and was therefore also log transformed.

3. Results

3.1. Habituation

Overall, cage-reared birds ate significantly fewer worms than hens reared in the aviary (Wald-$\chi2$ (1) = 10.62, $p < 0.001$, see Fig. 4A, N = 64). The interaction between whether the hen was selected for the holeboard test or not and the habituation day was significant, with selected hens starting with a higher number of worms eaten (Wald-$\chi2$ (3) = 9.50, p = 0.02, Fig. 4B, N = 64).

For both rearing environments, hens which were selected for the holeboard test (N = 28) did not differ in the number of worms eaten (Wald-$\chi2$ (1) = 0, p = 1, Fig. 4B), and the number of worms they ate increased across habituation days (Wald-$\chi2$ (3) = 9.92, $p < 0.02$). Looking at birds which were not selected for the holeboard test (Fig. 4B, N = 36), cage-reared hens ate significantly fewer worms than hens reared in the aviary (Wald-$\chi2$ (1) = 7.63, $p < 0.01$). The number of worms eaten increased for hens from both rearing conditions across habituation days (Wald-$\chi2$ (3) = 61.77, $p < 0.001$).

3.2. Holeboard test

Statistics from the holeboard test are summarised in Table 1. Data from the memory ratios are summarised in Fig. 5 and data from the time variables are summarised in Fig. 6.

3.2.1. Memory ratios

3.2.1.1. General working memory (GWM). The GWM ratio increased over time for cage- and aviary-reared hens during the Uncued phase ($F_{5135} = 7.12$, $p < 0.001$), the Cued phase ($F_{1,27} = 6.69$, $p < 0.015$) and the Reversal phase ($F_{2,52} = 6.39$, $p < 0.003$). The performance decreased during the transition between the Cued and Over-training phases ($F_{1,27} = 4.23$, $p < 0.05$, Fig. 5A), and between the Over-training and Reversal phases ($F_{1,27} = 11.02$, $p < 0.003$) for both treatment groups. No effects of the rearing environment were observed ($p > 0.05$, see Table 1).

3.2.1.2. Working memory (WM). The WM performances increased over time for both rearing environment during the Uncued and Cued phases ($F_{5135} = 4.63$, $p < 0.001$; $F_{1,27} = 6.78$, $p < 0.015$, respectively, Fig. 5B). No effects of the rearing environment were observed (see Table 1).

3.2.1.3. Reference memory (RM). For both cage- and aviary-reared hens, the RM ratio increased during the Uncued phase ($F_{5135} = 3.06$, $p < 0.012$) and the transition between the Uncued and Cued phases ($F_{1,27} = 27.95$, $p < 0.001$, Fig. 5C). The RM performances decreased during the transition between the Cued and Over-training phases ($F_{1,26} = 12.10$, $p < 0.002$) and between the Over-training and Reversal phases ($F_{1,27} = 13.87$, $p < 0.001$). The interaction between the rearing environment and the trial blocks was significant for the Cued phase ($F_{1,26} = 4.21$, $p < 0.05$), with hens reared in cages starting with a lower RM ratio than the aviary-reared hens but both groups reaching the same ratio at the end of the phase.

3.2.2. Time variables

3.2.2.1. Trial duration. Across time, hens from both rearing conditions became quicker at completing the task as shown by a decrease in trial duration during the Uncued phase, the Cued phase, and the Reversal phase ($p < 0.05$, see Table 1, Fig. 6A). The trial duration increased during all transitions between phases ($p < 0.05$, see Table 1). The cage-reared hens were slower than aviary-reared hens to complete the task during the transition between the Uncued and Cued phases ($F_{1,26} = 4.61$, $p < 0.02$).

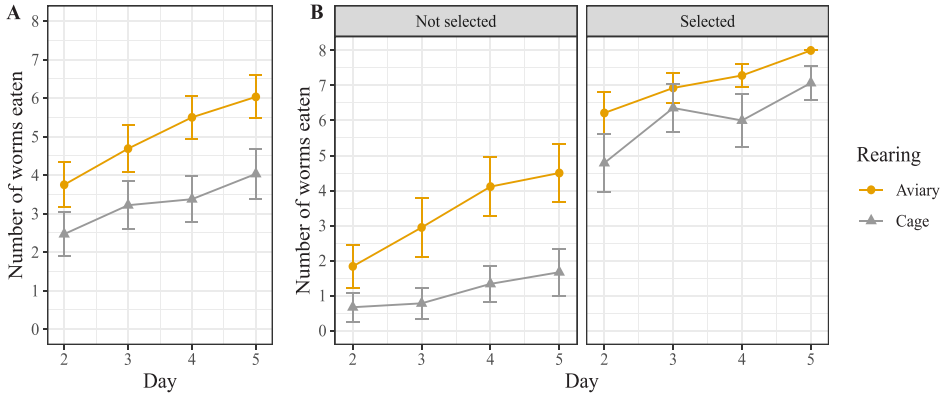

Fig. 4. Total number of worms eaten during the habituation phase (**A**) and number of worms eaten by the hens selected or not for the holeboard test (**B**) for each rearing environment. The graph shows the mean ± se.

Table 1
Results of linear mixed-effects models for all phases and transitions in the holeboard test. P-values were calculated by F tests.

	Rearing			Trial Block			Rearing x Trial Block		
	F	*df*	p≤	*F*	*df*	p≤	*F*	*df*	p≤
GWM									
Uncued	0.02	1,26	0.88	**7.12**	**5,135**	**< 0.0001**			n.s.
Trans. Uncued-Cued	0.18	1,26	0.68	0.47	1,27	0.50			n.s.
Cued	0.04	1,26	0.84	**6.69**	**1,27**	**0.02**			n.s.
Trans. Cued-Over Training	0.10	1,26	0.76	**4.23**	**1,27**	**0.05**			n.s.
Over Training	0.89	1,26	0.36	0.17	1,27	0.67			n.s.
Trans. Over Training-Reversal	0.00	1,26	0.98	**11.02**	**1,27**	**0.003**			n.s.
Reversal	2.04	1,26	0.17	**6.39**	**2,52**	**0.003**			n.s.
WM									
Uncued	1.07	1,26	0.31	**4.63**	**5,135**	**0.0006**			n.s.
Trans. Uncued-Cued	0.00	1,26	0.96	*3.86*	*1,27*	*0.06*			n.s.
Cued	0.20	1,26	0.66	**6.78**	**1,27**	**0.01**			n.s.
Trans. Cued-Over Training	0.12	1,26	0.73	1.61	1,27	0.22			n.s.
Over Training	0.11	1,26	0.74	0.00	1,27	0.99			n.s.
Trans. Over Training-Reversal	0.54	1,26	0.47	1.50	1,27	0.23			n.s.
Reversal	0.06	1,26	0.81	*2.45*	*2,52*	*0.10*			n.s.
RM									
Uncued	0.00	1,26	0.95	**3.06**	**5,135**	**0.01**			n.s.
Trans. Uncued-Cued	1.48	1,26	0.24	**27.95**	**1,27**	**< 0.0001**			n.s.
Cued	0.74	1,26	0.40	0.22	1,26	0.65	**4.21**	**1,26**	**0.05**
Trans. Cued-Over Training	0.53	1,26	0.47	**12.10**	**1,26**	**0.002**	*3.83*	*1,26*	*0.06*
Over Training	1.70	1,26	0.20	1.15	1,27	0.29			n.s.
Trans. Over Training-Reversal	0.00	1,26	0.98	**13.87**	**1,27**	**0.0009**			n.s.
Reversal	2.62	1,26	0.12	2.22	2,52	0.12			n.s.
Trial Duration									
Uncued	*2.94*	*1,26*	*0.10*	**27.45**	**5,135**	**< 0.0001**			n.s.
Trans. Uncued-Cued	**4.61**	**1,26**	**0.04**	**4.90**	**1,27**	**0.04**			n.s.
Cued	2.61	1,26	0.11	**36.15**	**1,27**	**< 0.0001**			n.s.
Trans. Cued-Over Training	0.72	1,26	0.40	**6.80**	**1,27**	**0.01**			n.s.
Over Training	0.41	1,26	0.53	0.02	1,27	0.90			n.s.
Trans. Over Training-Reversal	0.64	1,26	0.43	**10.88**	**1,27**	**0.003**			n.s.
Reversal	0.22	1,26	0.64	**4.10**	**2,52**	**0.02**			n.s.
Latency 1st bait									
Uncued	**5.26**	**1,26**	**0.03**	**8.21**	**5,130**	**< 0.0001**	*2.05*	*5,130*	*0.08*
Trans. Uncued-Cued	**6.19**	**1,26**	**0.02**	*4.04*	*1,27*	*0.05*			n.s.
Cued	**6.32**	**1,26**	**0.02**	**21.12**	**1,27**	**0.0001**			n.s.
Trans. Cued-Over Training	**5.87**	**1,26**	**0.02**	0.32	1,27	0.58			n.s.
Over Training	**6.29**	**1,26**	**0.02**	0.15	1,27	0.70			n.s.
Trans. Over Training-Reversal	2.39	1,26	0.13	**6.19**	**1,27**	**0.02**			n.s.
Reversal	0.48	1,26	0.49	2.25	2,52	0.12			n.s.

Significant comparisons ($p < 0.05$) are written in bold. Tendencies ($0.05 < p < 0.1$) are written in italics. GWM: General Working Memory; WM: Working Memory; RM: Reference Memory; Trans.: Transition

3.2.2.2. Latency to visit the first baited cup. Hens from both rearing conditions became faster at finding the first bait during the Uncued phase ($F_{5130} = 8.21$, $p < 0.001$) and the Cued phase ($F_{1,27} = 21.12$, $p < 0.001$, Fig. 6B). The latency to find the first bait increased during the transition between the Over-training and reversal phases ($F_{1,27} = 6.19$, $p < 0.02$). For the Uncued, Cued and Over-training phases and the transitions between them, aviary-reared hens were significantly faster to find the first bait than cage-reared hens ($p < 0.05$, see Table 1).

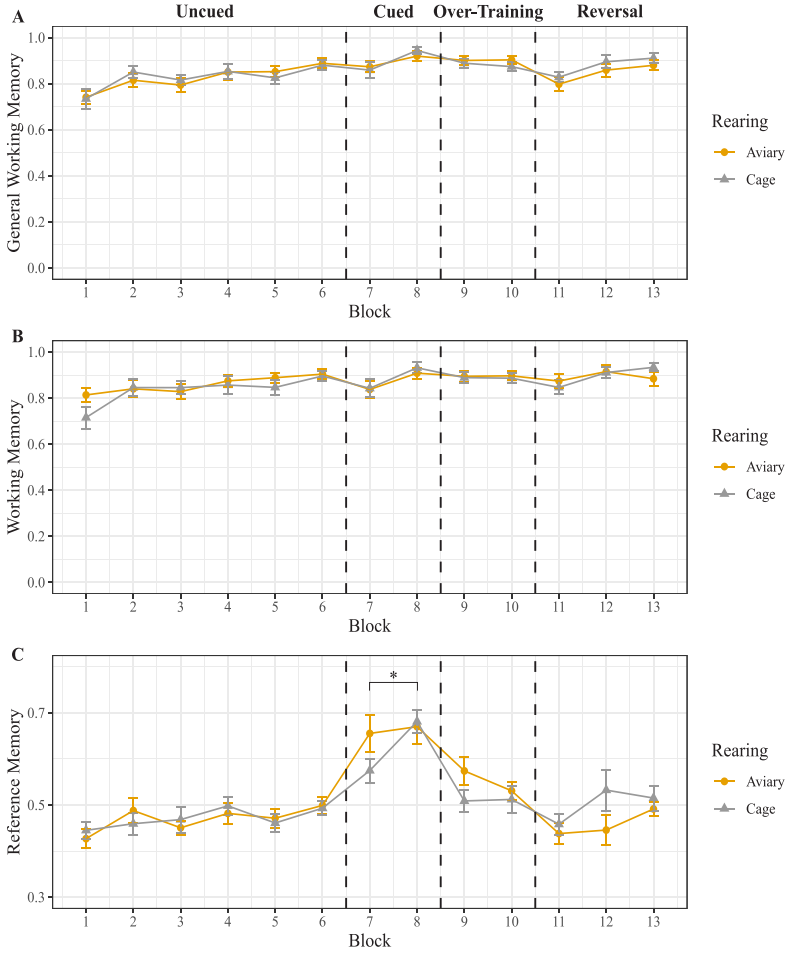

Fig. 5. Memory ratios from the holeboard test with the General Working Memory (**A**), the Working Memory (**B**) and the Reference Memory (**C**). A higher score indicates better memory performance. Trial blocks were averaged over two days of testing, except for block 12 which is the average of only the third testing day of the reversal phase (i.e., the average of two trials). The graphs show the mean ± se. * Indicates a significant interaction ($p < 0.05$).

4. Discussion

The aim of this study was to investigate whether the complexity of the rearing environment had medium-term effects on laying hens' spatial cognition. We predicted that due to a higher environmental complexity in the aviary, aviary-reared birds would show higher performances in the holeboard test compared to cage-reared birds.

4.1. Acquisition of the task

The results show an increase over time in general working memory (GWM) and working memory (WM) for both groups during the Uncued and Cued phases of the holeboard, reflecting that the birds revisited fewer cups with more experience of the task. This suggests that the birds got habituated to the arena and became more efficient at navigating it. This is supported by the fact that trial duration and the latency to the first baited cup also decreased over time, showing the birds were quicker to perform the task and to find the first bait. These results also support the idea that food deprivation prior to testing is not necessary for laying hens when the food reward is attractive (Arts et al., 2009; Nordquist et al., 2011; Tahamtani et al., 2015).

Previous studies on laying hens report WM ratios ranging between 0.7 and 0.8 (Nordquist et al., 2011; Tahamtani et al., 2015). The hens in our study demonstrated slightly higher WM ratios (0.8–0.9). This could be explained by differences in the study design. Indeed, Nordquist et al. (2011) and Tahamtani et al. (2015) tested the hens in a 3 × 3 matrix of cups, with three cups baited out of the nine. In the present study, we used a 2 × 4 matrix, with three cups baited out of the eight due to constraints in the space available to build the arenas in the henhouse. This design might be slightly simpler to navigate for the birds, which could explain the higher memory ratio.

Reference memory (RM) is usually used as an index to assess the ability of the individual to discriminate between baited and non-baited cups (van der Staay et al., 2012), and it reflects memory of the position of the baited cups across trials. In our study, RM ratios stayed relatively low (0.4–0.7) during all phases, though we observed an increase across time. These results are comparable to the ones obtained in previous studies on chickens (Nordquist et al., 2011; Tahamtani et al., 2015), but remained lower to the ones obtained in some studies on pigs (over 0.8 in Gieling et al., 2013; Grimberg-Henrici et al., 2016). It could be that birds did not learn the position of the baits and encountered them by chance while exploring the arena. Alternatively, it could be due to the fact that

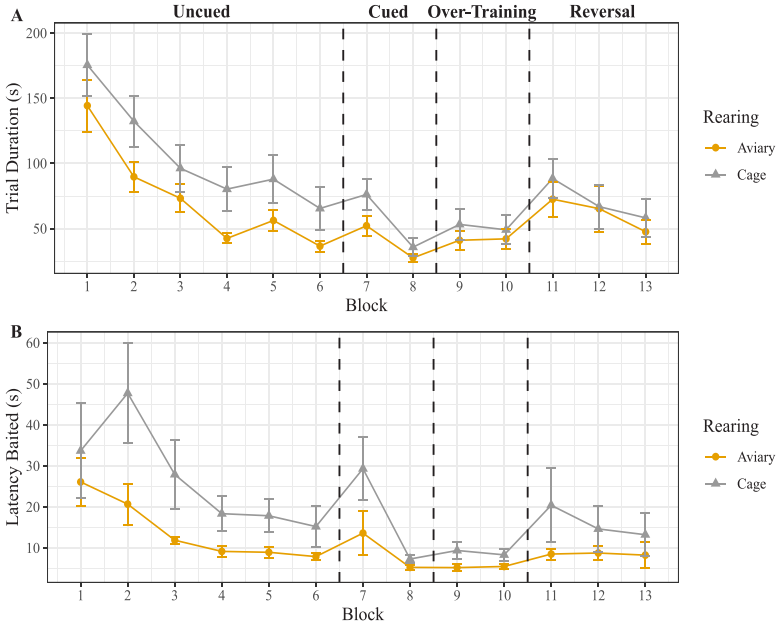

Fig. 6. Time variables from the holeboard test with the trial duration (**A**) and the latency to find the first bait (**B**). Trial blocks were averaged over two days of testing, except for block 12 which is the average of only the third testing day of the reversal phase (i.e., the average of two trials). The graphs show the mean ± se.

checking non-baited cups when moving from one baited cup to another is not costly. This second suggestion is supported by the drops in GWM and RM observed between the Over-Training and Reversal phases. Between those two phases, the configuration of baited cups of each bird is replaced by a new one, with no other changes in the arena. The GWM reflects the ability of the birds to avoid revisiting already visited cups (van der Staay et al., 2012). The drop in GWM between the Over-Training and Reversal phases thus indicates that the birds revisited cups more during the first block of the Reversal phase than during the last block of the Over-Training phase. It thus suggests that birds learned the position of the baits, at least to some extent. The drop in RM ratio suggests that birds revisited more unbaited cups during the Reversal phase than at the end of the Over-Training phase. They possibly revisit the locations of the baits from the previous configuration, but further analysis would be needed to confirm or invalidate this suggestion. To get clearer results on whether the birds discriminate between baited and non-baited cup, it could be interesting to increase the cost of visiting each cup. Indeed, White Leghorn hens have been shown to perform less contrafreeloading (i.e., choosing to forage over free food) than red jungle fowls (Jensen et al., 2002), and to get the majority of their food from the easily accessible site when offered the choice with a site requiring foraging (Schütz and Jensen, 2001). These findings suggest the hens might be more selective in their visits to the cups if they have to produce an effort to get access to the potential reward. This could be achieved, for example, by adding a swing lid to the cups so the hen has to dip its head inside the cup to check for food rewards.

4.2. *Effects of the rearing environment*

Overall, the rearing environmental complexity had few effects on the memory ratios measured during the holeboard test. This could be partly due to the fact that we selected the birds performing best at the end of the habituation phase to be tested in the holeboard task. To be able to assess the cognitive abilities of the birds, we had to select birds which were able to perform the task. In other words, birds which were actively exploring the arena and consuming rewards. The results from the habituation phase reflect that selection process, with birds which were selected for the holeboard test consuming more worms than birds which were not selected. Looking at the non-selected birds, there is a clear difference between the cage- and aviary-reared birds in the number of worms consumed. For each day of the habituation phase, cage-reared birds performed more poorly than aviary-reared birds and showed very low levels of worms eaten, despite five days of habituation. This could be either due to higher fear levels or lower cognitive abilities of the cage-reared birds. Previous research has demonstrated that increased environmental complexity during rearing reduced fearfulness in laying hens (Brantsæter et al., 2016; Nazar et al., 2022), which supports the idea that cage-reared hens might be more fearful. We saw that cage-reared birds started the Cued phase with a lower RM score than aviary reared birds but reached the same score by the end of the phase. This difference could reflect a stronger neophobic reaction to the introduction of cues (red plywood squares) for the cage-reared birds than for the aviary-reared birds. In contrast, results from our previous work showed that fear levels of birds reared in cages were comparable to the ones of aviary-reared birds when tested in an open field and a novel object test between 31 and 33 weeks of age (Dumontier et al., 2022). It seems that exposure to novelty alone in the arena (i.e., coloured cues) triggered a stronger neophobic reaction than the group exposure to a novel object in the cage (cup), and cage-reared hens seem to be more sensitive to changes in the environment than aviary-reared hens.

Tahamtani et al. (2015) reported a difference in working memory between hens reared in cages or in an aviary during the reversal phase. The results in our study do not present the same trend and the aviary- and cage-reared hens performed quite similarly. The hens tested in Tahamtani et al. (2015) were up to 23 weeks of age, whereas in our study they were up to 40 weeks of age. Those 17 additional weeks of housing in furnished cages at the laying farm could have evened out the effects of the rearing environment on cognition. A similar acclimatization to the laying environment has been observed by Pullin et al. (2020). Hens reared in barren cages showed a higher number of collisions than

aviary-reared hens when transferred to an enriched colony, but the differences between the two groups dissipated by 49 weeks of age. This suggests that the rearing environment has initial effects on behaviour, but that the effects eventually fade over time. However, it is important to note that the study by Tahamtani et al. (2015) and ours differ on some aspects of the experimental design (matrix of cups, randomisation of the position of the start box) which also could have affected the performance of the hens.

Despite the effect of the selection process on the results, we still notice differences between the two treatment groups on the latency to find the first bait. This indicates a strong effect of the rearing environment as despite selecting the best birds to be tested, the two groups behaved differently. Hens reared in the aviary were faster to find the first bait than the hens reared in cages for the Uncued, Cued, Over-Training phases and the transition between them. However, no differences in the trial duration were found between the two groups, except for the transition between the Uncued and Cued phase. That difference in trial duration might be explained by the potentially stronger neophobic reaction of the cage-reared hens to the cues when first exposed to them, as previously discussed. The difference in latency to find the first bait might reflect a lower motivation to start the trial from the cage-reared hens, or a stronger reluctance to leave the start area and explore the arena.

5. Conclusion

Overall, the rearing environment had little effect on the cognitive performances of the hens selected to be tested in the holeboard task. Though few differences were observed between the groups, the results from the habituation phase show that cage-reared hens eat significantly fewer worms than aviary-reared hens. The selection process prior to testing might have evened out the potential differences between the two rearing environments. However, despite the effects of the selection process, cage-reared hens were slower to find the first bait than aviary-reared hens and seemed to be more sensitive to changes in the environment.

Ethical statement

The animals used in this study were enrolled in a larger project. An application for permission to perform the animal studies was approved by the Norwegian Food Safety Authority (FOTS ID 22443). The experiments were performed in a farm approved as an experimental facility, and the experimental hens were housed in compliance with the Norwegian legislation regarding the use of research animals (Forskrift om bruk av dyr i forsøk). Animals were always handled gently to minimize stress and only healthy animals were included in the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Paper III

Environmental complexity and fearfulness are associated with spatial cognition and hippocampal plasticity in laying hens

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Abstract

Chronic stress negatively affects welfare, and laying hens are exposed to a variety of stressors throughout their lives. The environment experienced during early life can have long-lasting effects on the way individuals respond to stressors, but most work focuses on short-to medium-term effects. We therefore studied the effects of environmental complexity during rearing and the production period on chronic stress and spatial cognition in adult laying hens. We compared the ability of the individuals to perform a detour task and quantified hippocampal plasticity levels in the rostral and caudal subregions as a measure of chronic stress. We also studied the relationship between hippocampal plasticity and cognitive abilities, and related these measures to the distance walked in an open field arena as a measure of the birds' fearfulness. Hens were more likely to perform the detour during their second exposure and cage-reared hens were less likely to complete the task than aviary-reared hens. Distance walked in the open field arena was positively associated with the likelihood of performing the detour. Provision of enrichment in adult housing affected plasticity in the two subregions of the hippocampal formation (HF) differently for birds reared in cages or in an aviary. Provision of adult enrichment increased the cell density in the caudal HF for aviary-reared birds, while the effect was observed in the rostral HF for cage-reared birds. The multipolar cell density in the caudal HF was significantly higher in birds making the detour compared to the ones not making it, and in birds walking more during the open field test. The early environment thus seems to influence both cognitive test performance and hippocampal plasticity, with consequences for the effects of enrichment on hippocampal plasticity. Results from this study also highlight the role of underlying fearfulness in cognitive testing outcomes and hippocampal plasticity.

1 Introduction

Chronic stress has a negative effect on the organism with increased display of anxiety and decreased cognitive performance as potential outcomes (Eiland and McEwen, 2012). Though the response to an acute stressor is thought to be adaptive, as it protects the individual from danger, repeated exposure and disproportionate responses can have negative effects on welfare (McEwen, 2006). Repeated exposures to stressors lead to prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, which potentially, but not necessarily, leads to a higher baseline secretion of glucocorticoids (Herman et al., 2016; Gormally and Romero, 2018; Gualtieri et al., 2019). Exposure to glucocorticoids can, in the long-term, change the expression of glucocorticoid and mineralocorticoid receptors in the brain, affecting the negative feedback system and potentially decreasing HPA-axis sensitivity (Jacobson, 2005; Dickens et al., 2009). In addition, exposure to repeated stressors can affect the immune response, making individuals more vulnerable to pathogens (Nazar and Marin, 2011; Luo et al., 2020).

In the egg industry, birds are exposed to a variety of potential stressors. From being transported at one day of age to rearing facilities and later to production farms, to the exposure to constant noise and high bird densities, life in an industrial environment involves exposure to many challenges. In cage systems, the birds face limited opportunities to express highly motivated behaviours such as dustbathing, flying and wing flapping, and access to litter is restricted (Lay et al., 2011; Schuck-Paim et al., 2021b). In alternative housing such as aviaries or free-range systems, stress could be induced by housing in large social groups. Birds may also be exposed to higher risks of keel bone damage (Lay et al., 2011). Keel bone fracture is known to be painful for the hens and has been shown to induce a depressive-like state (Armstrong et al., 2020).

Excessive exposure to aversive experiences during early stages of life lead to individuals which are less stress resilient and more inclined to suffer from depressive disorders (Heim and Nemeroff, 2001; Pryce et al., 2005). For laying hens, this means the rearing period is a crucial phase. Brantsæter et al. (2016) showed that rearing hens in a low complexity environment led to young hens with higher fear levels

compared to hens reared in a more complex environment. Chicks exposed to standard hatchery procedures had higher stress responses than control chicks, and long-term effects were observed on the plumage quality and the occurrence of comb injuries (Hedlund et al., 2019). This highlights the importance of experience during early life on laying hens' stress responsivity and welfare.

However, the experience of chronic stress might be hard to quantify through observation of animal behaviour, and physiological markers do not necessarily reflect the same experience for each individual. For example, there is a complex relationship between the secretion of glucocorticoids and the individual experience, as glucocorticoids can be released both during negative and positive experiences (Chen et al., 2017). Adult hippocampal neurogenesis (AHN) is however known to be downregulated by chronic stress in rodents (Yun et al., 2010; Alves et al., 2018) and upregulated by positive experience such as enrichment and physical exercise (van Praag et al., 2000; Olson et al., 2006). Both the downregulation in negative conditions and the upregulation in positive ones depend on higher glucocorticoid titres (Lehmann et al., 2013). The former has been confirmed in poultry in recent studies, with birds undergoing unpredictable chronic mild stress showing lower levels of AHN than control hens (Gualtieri et al., 2019). Likewise, birds with severe keel bone fractures, a known cause of poor welfare in layers, also showed lower AHN levels than birds with minimal keel bone fractures (Armstrong et al., 2020). The hippocampal formation (HF) of birds can be divided into two subregions: the rostral and the caudal HF, respectively hypothesized to be homologous to the dorsal and the ventral hippocampal subregions in rodents (Smulders, 2017). These two subregions are thought to be involved in different processes, with the rostral HF being involved in spatial cognition whereas the caudal HF is sensitive to emotional stimuli and important in regulating the activity of the HPA axis (Smulders, 2017). AHN has recently been validated as a marker of cumulative affective experience (Poirier et al., 2019) and is a promising method for evaluating the effects of different stressors and production systems on laying hens' long-time welfare. Chronic stress could negatively impact AHN both in the rostral and caudal HF with potential consequences for spatial learning and for regulation of the HPA axis.

Cognitive abilities are known to be affected by chronic stress, fearfulness, and anxiety (Conrad, 2010; Sandi, 2013). Tree shrews which were exposed to chronic psychosocial stress showed altered declarative memory processes (Ohl and Fuchs, 1999), and rats exposed to maternal separation showed impaired spatial memory in a novel object recognition test (Eiland and McEwen, 2012). Previous studies also demonstrated that rats exposed to repeated subordination stress had impaired reference memory and were slower to learn a cognitive task than control rats (Krugers et al., 1997). In hens, fearfulness negatively affected learning and judgement bias (de Haas et al., 2017a; de Haas et al., 2017b). Similarly, no differences between enriched and barren housed rats were observed in the Morris water maze once wall hugging, a measure of behavioural anxiety, was taken into account (Harris et al., 2009). In addition, the environmental complexity experienced during early-life has been shown to impact cognitive abilities in a wide range of species, including in laying hens. For example, birds reared without early access to perches were less apt at reaching food placed in upper tiers at 19 weeks of age than birds with early-access to perches (Gunnarsson et al., 2000). Similarly, it has been shown that rearing in a barren environment negatively impacts spatial cognition up to seven weeks after transfer to the laying farm (Tahamtani et al., 2015). However, there is little evidence on how environmental complexity at different life-stages can affect cognition in the longer term, and to our knowledge, no studies have combined cognitive testing and hippocampal plasticity quantification in laying hens.

We here study the long-term effects of the rearing environmental complexity and adult environmental enrichment on spatial cognition and hippocampal plasticity, and how both relate to fearfulness levels. We tested hens in a detour task at the end of the production period (over 62 weeks of age) and collected their brains following the test to quantify doublecortin positive (DCX+) cells density in the hippocampal formation. In birds, DCX is still controversial as a marker of neurogenesis as it could also be expressed by neurons with high plasticity (Vellema et al., 2014). In a conservative approach, we will refer to AHN as hippocampal plasticity. We also studied the relationship between the detour test and hippocampal plasticity results and a measure of the birds' fearfulness, the distance walked in an open

field arena (Dumontier et al., 2022). We predicted that hens reared in the aviary would be more likely to perform the detour than cage-reared hens and that the provision of environmental enrichment would increase the number of hens making the detour regardless of rearing treatment. As individuals reared in enriched and complex environments are more resilient to stressors than birds reared in less complex environments (Hegde et al., 2020), we predicted that hens reared in the aviary would show higher levels of hippocampal plasticity than hens reared in cages. We also predicted that the provision of environmental enrichment would increase hippocampal plasticity.

2 Methods

2.1 Animals, rearing and housing

2.1.1 Rearing

This study was conducted on non-beak trimmed White Leghorn hens (N = 64). These hens were enrolled in a larger project for which 384 hens were reared either in a cage (N = 192) or in an aviary (N = 192) before being transferred to the experimental farm at 18 weeks of age. The birds were all reared at a commercial hatchery (Steinlands & co., Norway) in the same room. The room measured 15 m x 72 m and contained 38,000 birds housed in a raised NATURA Primus 16 system (Big Dutchman, http://www.bigdutchman.com/). The system consisted of three tiers measuring each 12 m x 0.8 m x 0.6 m (length x height x width). The front of each tier could be either closed or open to manage access of the birds to the different tiers of the system and to the floor of the house. Each tier was furnished with a feedline, nipple drinkers and perches above the feed and water lines. After hatching, birds were placed on the first and second tiers of the system. The mesh floor of the aviary rows was lined with paper until 4 weeks of age. For birds reared in the cage condition, the front of the tier was kept closed for the whole rearing period. These birds were situated in the second tier of one of the aviary rows, the tier containing 250 birds. For all other birds, the front of the tier was opened from five weeks of age. The birds could move freely between the different tiers of the aviary rows and access the floor of the house. Wood shavings were used on the floor of the house as litter material. Once the front of the

cage was open, birds had access to perches on the front of each tier of the aviary row, and additional perches were extended from the cage front at seven weeks of age.

From 5 weeks of age, the density was 26 birds/m^2 for the cage-reared birds and 29 birds/m^2 for the aviary-reared birds. Before releasing the aviary-reared birds in the room, the stocking density was the same for both treatment groups (245 birds/m^2). All birds were exposed to the same lighting and feeding schedule. Temperature started at 34 °C and was gradually decreased to 19 °C at 16 weeks of age. Birds were exposed to 24 h of light for the first day, followed by a continuous 4:2 light/dark cycle during the first week as recommended by the Lohman LSL management guide. The light schedule was then switched to 16:8 light/dark at two weeks of age and gradually decreased to 9:15 light/dark by five weeks of age. Gradual transitions from dark to light and from light to dark were used. Each transition took 20 min. All birds received vaccination against coccidiosis and Marek's disease.

At 18 weeks of age, 192 birds were randomly selected from the aviary (aviary-reared birds) and 192 birds were randomly selected from the tier which was kept closed (cage-reared birds).

2.1.2 Adult housing at the experimental farm

At 18 weeks of age, birds were transferred to the experimental farm. They were all housed in the same room containing 2808 cages organised in 12 rows. Each row contained six tiers, with a walkway between the 3rd and 4th tier forming the second floor of the henhouse. Experimental birds were all housed in the third tier of the second floor (i.e., the top tier). They were housed in social groups of four individuals from the same rearing environment in two Victorsson T10 furnished cages. The two cages were connected by an opening measuring 15 cm x 18 cm and measured in total 240 cm x 83 cm x 63 cm (width x height x depth). Each pair of cages containing four birds will hereafter be referred to as a cage. The distribution of treatments in cages was balanced so that cages with birds reared in the aviary were always next to cages with birds reared in cages.

Half of the birds used in this experiment were housed in standard furnished cages (N = 32, 16 from each rearing condition) and the other half in additionally enriched furnished cages (N = 32, 16 from

each rearing condition). Cages with additional enrichment was distributed in the hen house so that two enriched cages (one containing aviary-reared birds and one containing cage-reared birds) were always next to two standard cages. Standard furnished cages (N = 32) were furnished with two nest boxes, four perches and a dustbathing tray on the roof of each nest box (see Fig. 1A). Additionally enriched cages (N = 32) were the same as the standard furnished cages with the provision of an additional dustbathing platform (55 cm × 60 cm width × depth with a 2 cm high frame), a hemp pompon to peck at, and polyethylene tarp curtains and sheets to increase environmental complexity (see Fig. 1B). The curtains hung under one of the perches and on the upper edge of the opening between the two parts of the cage. The additional dustbathing platform was placed on the perches in one half of the cage and refilled weekly with a mixture of feed crumbles and dustbathing pellets (wheat husks). The pompom hung from the cage front above the dustbathing platform. To keep the environment stimulating from 42 weeks of age, the dustbathing platform and pompom were switched to the other side of the cage every two months.

All birds were exposed to the same lighting and feeding treatment during their time at the farm. From their arrival at the experimental farm at the age of 18 weeks, they were kept under a 13:11 light/dark cycle and a temperature of 21.1 ± 1.6 °C. Gradual transitions from dark to light and from light to dark were used. Each transition took 15 minutes. Food and water were provided ad libitum via a food chain running in front of the cages and a water line with nipple drinkers along the back of the cages. For identification purposes, each bird was individually marked by means of a black or white plastic zip-tie around its left or right leg.

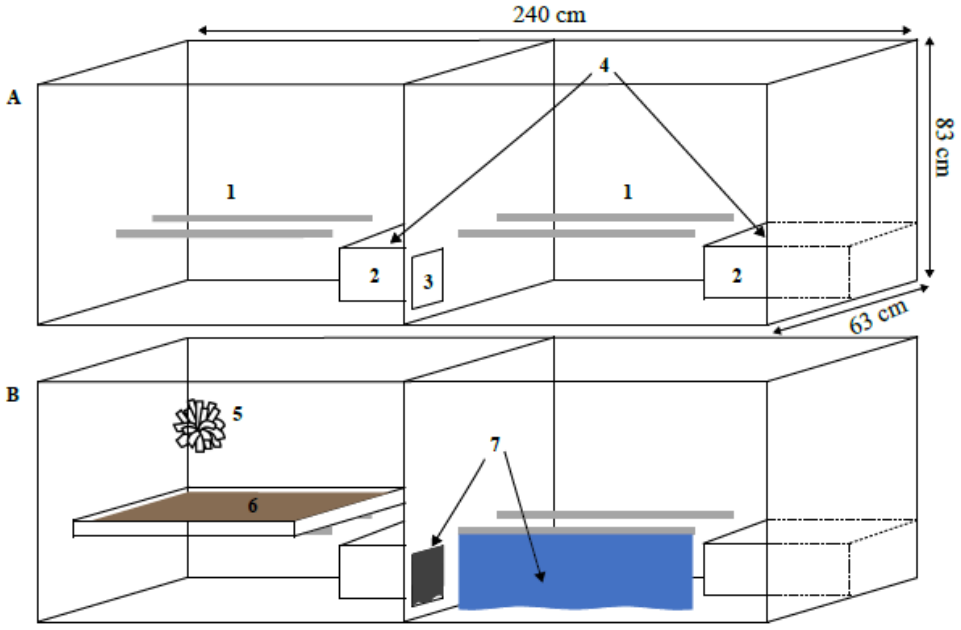

Figure 1: Schemas of a standard Victorsson T10 furnished cage (**A**) and an additionally enriched Victorsson T10 furnished cage (**B**), three-quarter front view, showing (**1**) the perches, (**2**) the nest boxes, (**3**) the opening between the two parts of the cage, (**4**) the dustbathing trays, (**5**) the hemp pompom, (**6**) the additional dustbathing tray and (**7**) the curtains. The features 1–4 were also accessible in the additionally enriched cages.

2.2 Behavioural tests

One bird per cage was tested twice in a detour task, once at 62 and once at 64 weeks of age (N = 16 per group). The tests were conducted in a temporary arena built in the hen house. The arena measured 350 cm x 177 cm and the walls were 190 cm of height. Three of the walls were made of wood frames covered with dark tarps, the fourth wall being the wall of the henhouse in grey concrete. The floor was made of particle boards, and lighting was provided by a lamp mounted on one of the walls. One camera (Axis m1124-e network camera, Noldus, The Netherlands) was set up on the concrete wall over the arena to record the trials. All trials were recorded using the MediaRecorder system (Noldus Information Technology, Wageningen, The Netherlands). The detour compartment

(60 cm x 60 cm x 60 cm) was made of solid walls and floor with a grid front and roof and an open rear end. It was placed in the arena facing a stimulus compartment (Fig. 2). The stimulus compartment measured 50 cm x 82 cm x 60 cm (width x length x height) and was made of solid walls and roof with a grid front. The stimulus compartment contained two familiar hens and a dish with food and live mealworms (known as a palatable food reward, Moe et al. (2009)). The familiar hens were chosen from the same cage as the tested hen. The tested hen was placed with the familiar hens for 1 min to increase motivation to join the cage mates and/or the food. One minute was enough for the hens to settle down and start eating. The tested hen was then placed into the detour compartment. The test lasted until the hen performed the detour, or until the maximum cut-off of 10 min has elapsed, whichever occurred first. The detour was considered performed when the hen walked past the front of the detour compartment, as shown by the dotted line in Figure 2. Walking past the front of the detour compartment was visually defined as when the centre point of the hen crossed the dotted line. The latency to perform the detour was manually scored for each trial.

The birds tested during this experiment were previously tested in a novel object and an open field test at 60-61 weeks of age. For a complete report of the results and detailed methods, see Dumontier et al. (2022). To include a measure of the hens' fearfulness in the analysis, the distance walked in the open field arena was used in this study as well. In many species, the distance walked in an arena is usually associated with the individual's fearfulness with individual walking more being typically less fearful (Forkman et al., 2007). Briefly, the open field test was conducted in the same arena as the detour test when the hens were 61 weeks of age. The tests were video recorded, and the videos were analysed using EthoVision X9 (Noldus Information Technology, Wageningen, The Netherlands). The distance walked in the arena by the hen was recorded by tracking the centre point of the hen during the 10 min duration of the test.

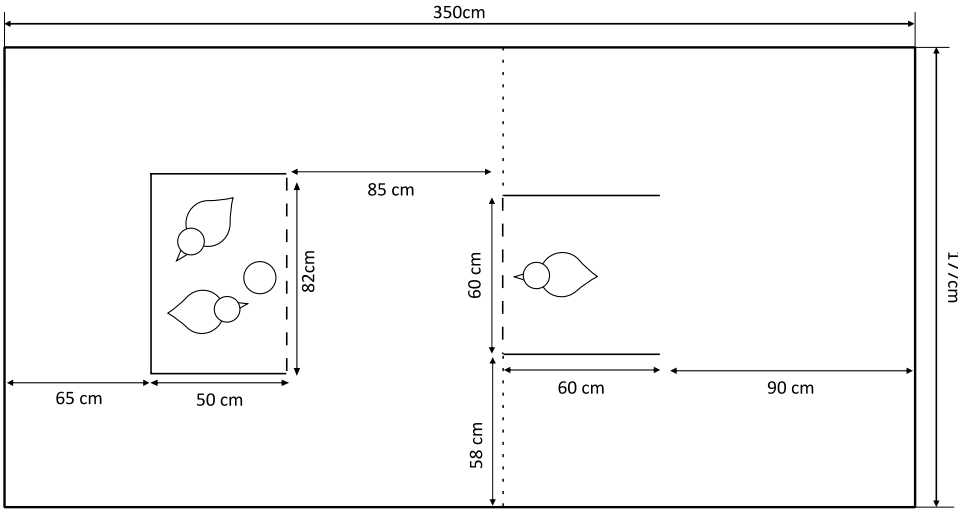

Figure 2: Schema of the detour arena, view from above. The stimulus compartment is shown on the left-hand side of the schema, and the detour compartment on the right-hand side. The detour was considered completed when the centre point of the hen crossed the thin dotted line.

2.3 Hippocampal plasticity

2.3.1 Tissue collection

The week following the last detour test, the brains from 12 birds from each of the four treatment groups were collected. The birds were randomly selected from each treatment group among the birds tested in the detour test. The birds were anesthetized using 0.5 ml/kg of an anaesthesia mixture consisting of an alfa-2 agonist (xylazine), an opioid (butorphanol), a dissociative anaesthetic (tiletamine) and a benzodiazepine (zolazepam) (10 ml Rompun vet. (Xylazine 20 mg/ml)), 0.75 ml Butomidor (Butorphanol 10 mg/ml) mixed with one vial of Zoletil vet. powder (Tiletamine HCL 125 mg, and Zolazepam HCL 125 mg) by intramuscular injection. When a deep plane of anaesthesia was reached and the bird did not respond to external stimuli, they were killed by cervical dislocation.

The brain was extracted from the skull and placed in a petri dish with saline solution. The two hemispheres were separated along the longitudinal fissure using a scalpel. The hippocampal formation (HF) of one of the hemispheres was dissected for further molecular analysis (results to be reported

elsewhere), while the other hemisphere was treated for immunohistochemistry analysis. The choice of hemisphere was balanced for each treatment group so that the same number of left and right hemispheres were treated for immunohistochemistry.

2.3.2 Immunohistochemistry

The whole hemispheres (N = 12 per group) were fixed in 4% paraformaldehyde in 0.1 M Phosphate Buffered Saline (PBS) at 4 °C for 48 hours. They were then transferred to a 30% sucrose in PBS solution for cryoprotection at 4 °C for 48 hours. The sucrose solution was renewed once during this time span. After 48 h, the brains had sunk to the bottom of the vials and the hemispheres were removed from the solution. In case the brains had not sunk to the bottom of the vials, they were left 24 extra hours in the sucrose solution. Excess fluid was removed, and each hemisphere was placed in a mould (70183, Electron Microscopy Science - USA) with the cut midline down, to be embedded in OCT (62550, Electron Microscopy Science – USA). The moulds were put in dry ice until the sample was frozen and wrapped in aluminium foil. Samples were kept at -80°C until further analyses. Before staining, samples were cut into 50 µm thick coronal sections using a cryostat (CM1860, Leica). The sections were temporarily stored in 0.1 M PBS (up to 72 h) before being stored at -20°C in cryoprotectant (30% glycerol and 30% ethylene glycol in 0.1 M PBS).

2.3.3 Free-floating sections protocol

Using a free-floating sections protocol, the sections were stained by immunofluorescence against doublecortin. Sections were pre-treated with a 30 min endogenous peroxidase inhibition (3.3% H_2O_2) for 30 min, washed (3 x 5 min) in 0.1M PBS, and incubated for 60 min in a blocking solution (2% goat serum, 1% Bovine Serum Albumin in 0.1 M PBS containing 0.3% Triton X-100.). Following a quick rinse in dH_2O, sections were incubated for 16 hours at 4°C in a 1:1000 polyclonal antibody raised in rabbit against DCX solution (Abcam ab18723). Sections were washed (3 x 5 min) in 0.1M PBS and incubated at room temperature for 120 min in a 1:2000 goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody solution (Alexa Fluor Plus 594- RED). Sections were washed (3 x 5 min) in 0.1M

PBS, stained with a 300 nM solution of DAPI and washed again in 0.1M PBS (3 x 5 min). Sections were sorted along the rostro-caudal axis, mounted on gelatin subbed slides and coverslipped with ProLong glass antifade mountant (Fisher, ref. P36984). For three of the brains, staining with DAPI was done on the slides after mounting. For all other brains, sections were stained directly into the wells before sorting and mounting.

2.3.4 Bioimaging and stereological quantification

For 6 birds per group, two rostral sections spaced by 800 µm and one caudal section were analysed. Rostral sections ranged between interaural 3.76 and interaural 2.32, and the caudal section was situated caudal of interaural 0.40 (Puelles et al., 2007). We used Zeiss AxioImager with Apotome systems and the Zen software to acquire images. The HF of each section was outlined at a x10 magnification based on the chick brain atlas and tiles measuring 332.8 µm x 332.8 µm spaced by 75 % were imaged at x40 magnification. A Z-stack of 19 layers, spaced by 0.90 µm were collected for each tile. Images were collected for the Alexa 594 channel and the DAPI channel.

Images were analysed with the Fiji software (Schindelin et al., 2012), using the cell counter plug-in. For each tile, the tissue area was measured and DCX+ cells were counted. They were split in two categories: bipolar cells, which are young migrating cells, and multipolar cells, which are older and settling. Bipolar cells were defined as small/medium size elongated cells with a maximum of two processes (Fig. 3). Multipolar cells were defined as medium/large cells with more than two processes (Fig. 3). For each identified cell, the presence of a nucleus was checked in the DAPI channel to confirm the identification. For each HF subregion (rostral or caudal), densities of each cell type in number of cells/μm^3 were calculated by dividing the number of cells by the total tissue volume sampled (area of the tissue multiplied by the section thickness (50 µm)). Densities were converted to number of cell/mm^3 by multiplying the densities in cell/μm^3 by 10^9.

All sections were analysed by the same experimenter (LD) who was blind to treatments.

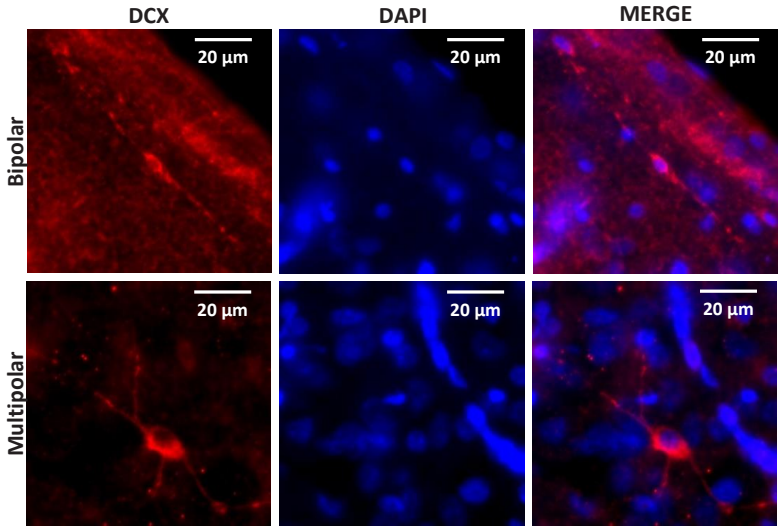

Figure 3: Representative images of a bipolar and a multipolar DCX+ cell taken at x40 magnification.

2.4 Data analysis

All statistical analyses were performed with R, version 4.0.3 (R Core Team, 2022). The individual was used as the statistical unit.

Latencies to perform the detour were analysed with an accelerated failure time model using the survreg function from the *survival* package (Therneau, 2020). The rearing environment, adult environment and round of testing were included as predictors. The Bird ID was included in the model as a cluster to account for repeated measures. This model was then expanded to include the distance walked during the open field test to study the relationship between the detour performance and the hens' fearfulness. We assessed the goodness of fit of different distributions by comparing their AIC and picked the lognormal distribution which showed the lowest AIC. P-values were calculated with Wald tests. Every two-way interaction was included in the models and removed when not significant.

Correlations between the cell densities in the rostral and caudal HF were calculated for each cell type (bipolar or multipolar) with a Pearson correlation test. The same test was used to study the correlation between bipolar and multipolar cell densities for each HF subregion. Differences in cell densities

between the rostral and caudal HF and between cell types (bipolar or multipolar) were analysed separately with a linear mixed effect model (LMM) using the R package *lme4* (Bates et al., 2015), with the Brain ID as a random effect. To study the effects of the environment, cell densities were analysed for each cell type (bipolar or multipolar) separately with a LMM. The HF subregion, rearing environment and adult environment were used as predictors. The Bird ID nested within the staining batch was used as a random intercept factor to account for differences in staining between batches and to correct for the same origin of the samples. Another model with the same random effect structure was run with the HF subregion and whether the bird performed the detour or not on their first exposure to the test. The aim was to study any difference in neuroplasticity between those birds who managed and those who did not manage the detour. As the HF is sensitive to emotional stimuli, we ran a similar model with the distance walked in the open field arena (details in (Dumontier et al., 2022)) instead of the detour performance as a measure of the hens' fearfulness to test the relationship between fearfulness and hippocampal plasticity. Every two-way interaction was included in the models and removed when not significant. All models were visually examined and complied with the assumptions for normality of residuals and homogeneity of variances. P-values were calculated by corrected F-tests with the Kenward-Roger approximation (Bolker et al., 2009).

2.5 Ethical statement

The animals used in this study were enrolled in a larger project. An application for permission to perform the animal studies was submitted to and approved by the Norwegian Food Safety Authority (FOTS ID 22443). The experiments were performed in a farm approved as an experimental facility, and the experimental hens were housed in compliance with the Norwegian legislation regarding the use of animals in research (Forskrift om bruk av dyr i forsøk).

3 Results

3.1.1 Detour test

We used an accelerated failure time model with the treatment groups during rearing and laying and the round of testing as predictors to determine whether the occurrence and latencies of detour events differed between the different treatment groups (Fig. 4). Aviary-reared hens were faster and more likely to perform the detour than cage-reared hens ($\chi^2 = 4.04$, df = 1, $p = 0.04$), but there was no main effect of enrichment provision during the production period ($\chi^2 = 1.36$, df = 1, $p = 0.24$). For all treatment groups, the hens were faster to perform the detour during the second exposure compared to the first one ($\chi^2 = 22.49$, df = 1, $p < 0.01$).

Results from the accelerated failure time model including the distance walked during the open field test showed a strong effect of the distance walked in the arena on the ability to perform the detour ($\chi^2 = 17.33$, df = 1, $p < 0.001$). The effects of the round of testing ($\chi^2 = 22.10$, df = 1, $p < 0.001$) and provision of enrichment ($\chi^2 = 0.03$, df = 1, $p = 0.85$) remained unchanged, while cage-reared hens tended to be less likely and slower to perform the detour than aviary reared hens ($\chi^2 = 2.84$, df = 1, $p = 0.09$).

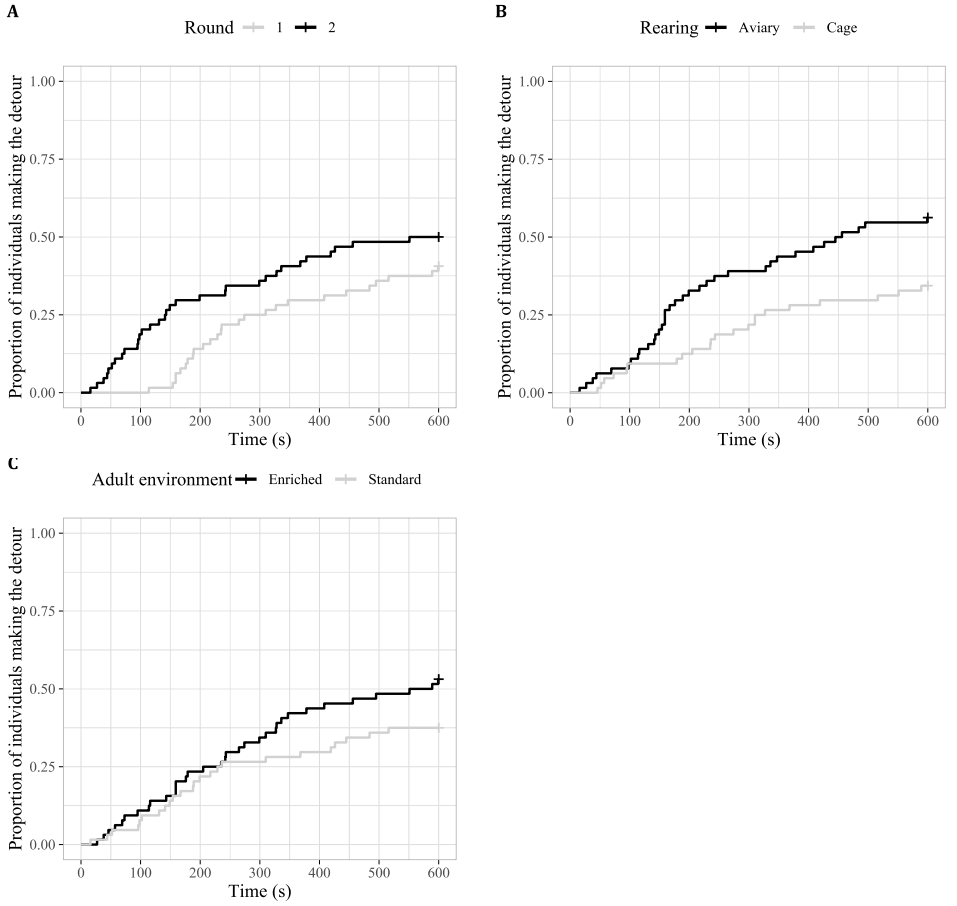

Figure 4: Kaplan-Meier curves showing the latency to make the detour for the two different exposures to the test (**A**) the two rearing environments (**B**) and the two housings during the production period (**C**).

326 *3.1.2 Hippocampal plasticity analysis*

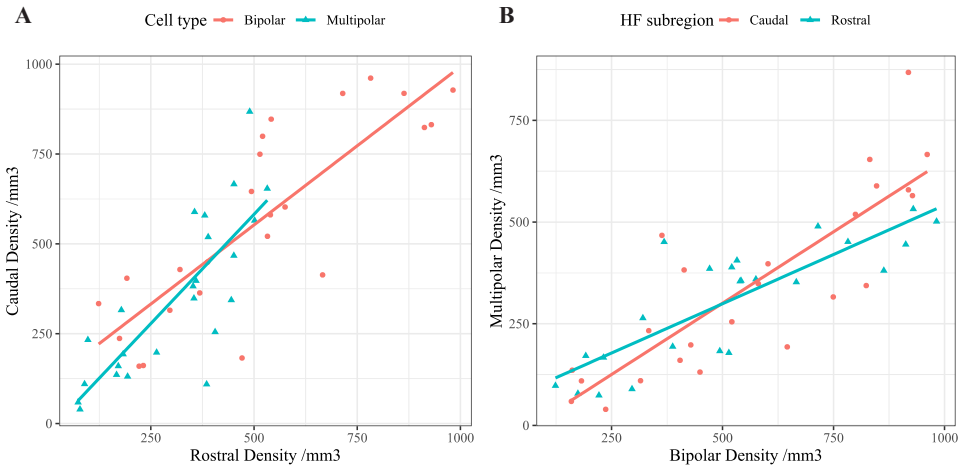

Figure 5: Cell densities correlations between the rostral and caudal HF for each cell type (**A**) and between bipolar and multipolar cells for each HF subregion (**B**). HF: Hippocampal Formation

327 There was a strong positive correlation between the cell density in the rostral and in the caudal HF
328 subregion for both cell types (bipolar: $R = 0.82$, $p < 0.001$; multipolar: $R = 0.80$, $p < 0.001$, Fig. 5A). The
329 bipolar cell density was strongly correlated with the multipolar cell density (Fig. 5B) in the caudal HF
330 ($R = 0.84$, $p < 0.001$) and in the rostral HF ($R = 0.83$, $p < 0.001$). The cell density did not differ between
331 subregions ($F_{1,71} = 1.76$, $p = 0.19$; rostral: mean ± SEM = 411 ± 33 cells/mm^3, caudal: 456 ± 39
332 cells/mm^3), but the density of bipolar cells (540 ± 38 cells/mm^3) was higher than that of multipolar
333 cells (326 ± 27 cells/mm^3; $F_{1,71} = 80.62$, $p < 0.001$).

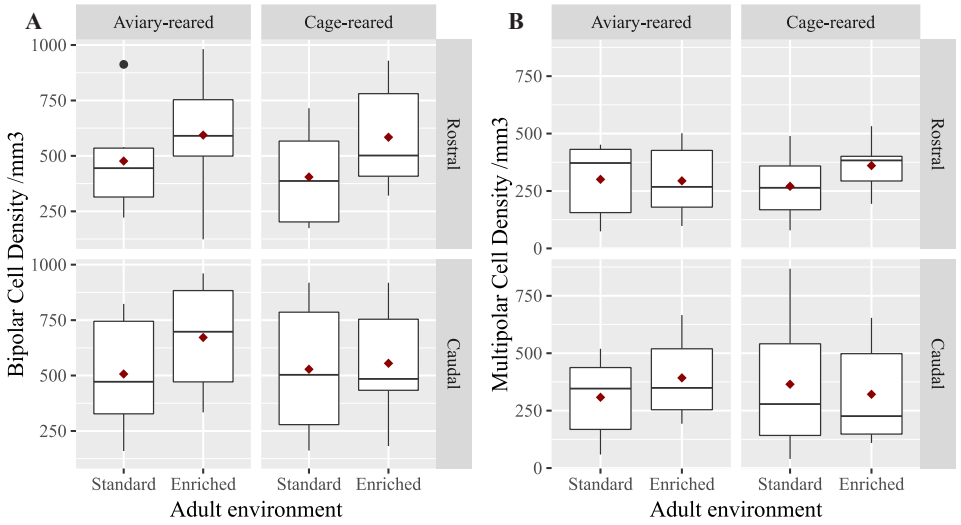

Figure 6: Densities of (**A**) bipolar and (**B**) multipolar cells in the rostral and caudal subregions of the HF for birds reared in cages or in an aviary and housed in standard or enriched furnished cages for the laying phase.

There was an interaction between the rearing environment, the adult environment, and the hippocampal subregion ($F_{1,20}$ = 4.24, p = 0.05) for the multipolar cell density (Fig. 6B). The effect of enrichment during the production phase increased the cell density in the caudal HF for aviary-reared birds, whereas the effect were observed in the rostral HF for cage-reared birds. Paired comparisons revealed no significant effects.

The rearing environment had no effects on the bipolar cell density ($F_{1,20}$ = 0.25, p = 0.61, Fig. 6A), neither had the provision of enrichment during the production phase ($F_{1,20}$ = 1.98, p = 0.17). Bipolar cell density did not differ between the two subregions ($F_{1,23}$ = 2.58, p = 0.12).

342 *3.1.3 Relationship between hippocampal plasticity and detour performances*

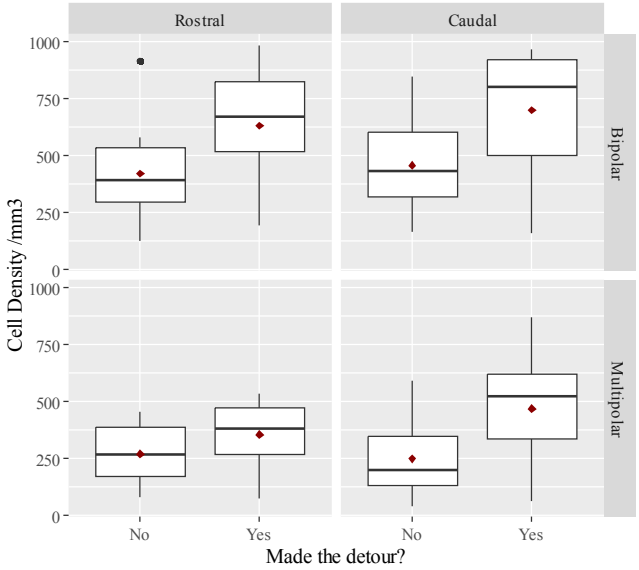

Figure 7: Densities of bipolar and multipolar DCX+ cells in the rostral and caudal HF for birds which made the detour or not during their first exposure to the test.

343 Bipolar cell density did not significantly differ between birds making the detour during their first
344 exposure to the task or not ($F_{1,21}$ = 3.74; p = 0.07), and there was no effect of the subregion ($F_{1,22}$ =
345 2.60; p = 0.12, Fig. 7)). There was an interaction between the subregion and the detour test outcome
346 for the multipolar cell density ($F_{1,22}$ = 7.06; p = 0.01), with hens making the detour having a higher cell
347 density in the caudal than in the rostral HF (t = -3.042, p = 0.006). The cell density in the caudal HF
348 differed significantly between birds making the detour compared to the one not making the detour,
349 with birds making the detour having a higher cell density (t = 2.63, p = 0.01). There were no effects of
350 the detour group ($F_{1,21}$ = 2.90; p = 0.10) or subregion ($F_{1,22}$ = 3.31; p = 0.08) alone.

3.1.4 *Relationship between hippocampal plasticity and fearfulness*

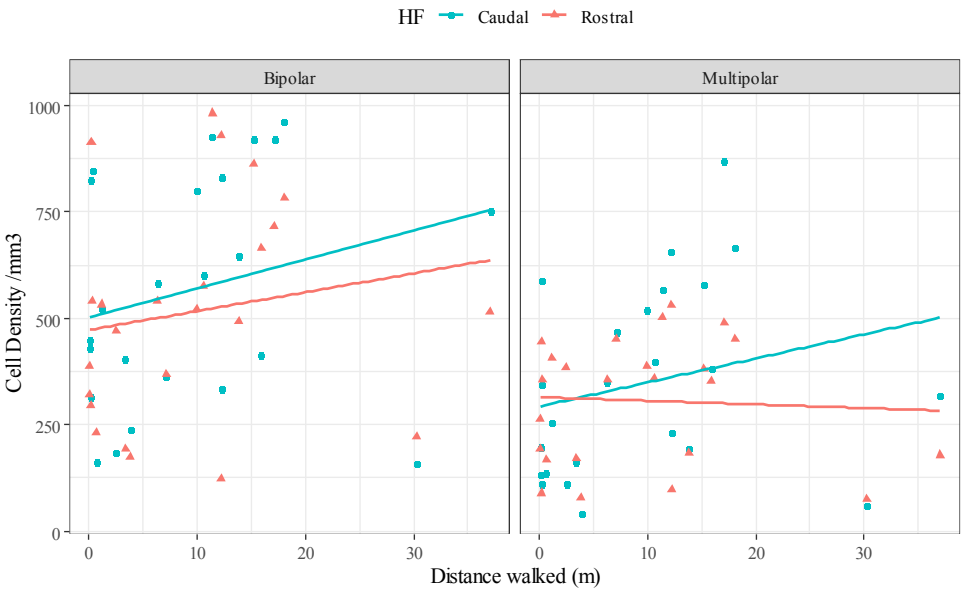

Figure 8: Plots showing the relationship between the distance walked during the open field test and the bipolar and multipolar cell densities in the rostral and caudal HF.

Regarding effects on hippocampal plasticity, there was a significant interaction between the distance walked during the open field test and the HF subdivision on the DCX+ multipolar cell density ($F_{1,22}$ = 5.71; p = 0.03, Fig. 8). Hens walking less during the open field test showed a lower DCX+ multipolar cell density in the caudal HF than hens walking more, while the cell density in the rostral HF was relatively constant. No other significant effects were reported on the distance walked ($F_{1,21}$ = 0.15; p = 0.70) or HF subregion ($F_{1,22}$ = 0.35; p = 0.56) alone. The DCX+ bipolar cell density was not significantly influenced by the distance walked in the open field arena ($F_{1,21}$ = 0.85; p = 0.37) nor the HF subregion ($F_{1,23}$ = 2.58; p = 0.12).

4 Discussion

The aim of the study was to investigate the effects of environmental complexity at different life stages on laying hens' spatial cognition and hippocampal plasticity. Results show that environmental complexity during rearing affected spatial cognition but had limited effects on HF plasticity, while fearfulness was strongly associated with detour performance. The link between HF plasticity and the ability to perform the detour or the distance walked in the open field arena indicated cell-type and subregion specific relationships.

Effects of the environment on spatial cognition

Environmental complexity is known to affect spatial cognition. For example, chicks housed with visual barriers had better cognitive abilities than chicks reared without, demonstrated by the smaller number of orientation errors in the detour test (Freire et al., 2004). Other studies suggested that housing chicks in environments with greater variety of perches and litters decreased the latency to perform a detour (Skånberg et al., 2023). Our results support these previous findings and suggest that the environmental complexity during rearing can have long-lasting effects on the ability to solve a detour test, at least until 64 weeks of age. The birds tested in this experiment were previously tested in an open field test (Dumontier et al., 2022). No differences were found between the treatment groups in terms of distance walked in the arena, suggesting the differences observed between the two rearing groups in the detour test are not due to locomotory impairments of cage-reared hens. The hens were also faster and more likely to perform the detour during their second exposure to the test than during the first one, suggesting habituation to the task. Contrary to our expectations, the provision of enrichment during the production period did not improve the performance in the detour test. It could be that the fact all hens could navigate between the two parts of the cage represented a source of environmental complexity relevant to the detour task. The addition of curtains as visual barriers in the additionally enriched cages might therefore not have represented a relevant increase in complexity.

Effects of the environment on hippocampal plasticity

Contrary to what has been found in previous studies (Gualtieri et al., 2019; Armstrong et al., 2022), we did not observe differences in DCX+ cell density between the rostral and the caudal HF. There was however a strong correlation in the cell density between the two subregions for both cell types. We also observed an overall higher density for DCX+ bipolar cells than multipolar cells, consistent with previous work (Armstrong et al., 2022).

The different environments during rearing and laying had little effects on the DCX+ bipolar cell density in both the rostral and the caudal HF, suggesting no effects on cell proliferation and/or maturation. There was, however, a significant three-way interaction between the environments during rearing, laying and the HF subregion for the multipolar cell density. The provision of enrichment during the production period affected the cell survival differently in the two HF subregions for each rearing environment. Though paired comparisons were not significant, providing environmental enrichment increased the multipolar cell density in the caudal HF for aviary-reared hens, while the effects were observed in the rostral HF for cage-reared hens. The caudal HF is sensitive to stress, which suggests housing aviary-reared hens without additional enrichment might induce a state of chronic stress. As the rostral HF is involved in spatial cognition, it seems the provision of environmental enrichment for cage-reared hens stimulated plasticity with regards to their cognitive abilities. Similarly, it has also been found that housing pigeons in enriched cages increased the density of DCX+ cells in the hippocampus (Melleu et al., 2016).

Overall, the effects of the different treatment groups on hippocampal plasticity in the current experiment were rather limited. It could be that the standard housing during the production period already offered enough opportunities to the hens, and that the added enrichment did not make enough of a difference. Indeed, the furnished cages used in our studies are designed to house up to 10 hens in industrial setups. In our experiment, four hens were housed in two communicating cages. It means they had access to two dustbathing platforms and four perches, which reduces the risk of

competition for these resources and, hence potentially diminished the experience of social stress (EFSA Panel on Animal Health and Animal Welfare, 2023). They also had access to more space per hen, enabling them to perform more behaviours such as wing flapping or stretching (Schuck-Paim et al., 2021a). These conditions could have been sufficient to reduce the experience of chronic stress. In addition, no actual stressors were applied to the birds at any stages of their life. In most studies assessing the effects of chronic stress on hippocampal DCX+ density, stressors are applied for an extended period of time. For example, Gualtieri et al. (2019) exposed hens to unpredictable chronic mild stress during several weeks. Other studies compared hens with severe keel bone damage to hens with minimal keel bone damage (Armstrong et al., 2020), or hens with poor body condition with hens with good body condition (Armstrong et al., 2022). All of these could probably be considered as more severe than exposure to low environmental complexity. Supporting this view, Armstrong et al. (2022) did not find differences in hippocampal plasticity between hens housed in furnished cages vs. free-range multi-tier cage-free systems, despite finding effects of the birds' body condition on hippocampal plasticity.

Relationship between hippocampal plasticity, detour performances and fearfulness

Looking at the relationship between the ability to perform the detour or not and the DCX+ cell density, it appeared that hens not making the detour showed a lower multipolar DCX+ cell density in the caudal HF than hens making the detour. Considering the caudal HF is thought to be homologous to the ventral mammalian hippocampus and sensitive to emotional stimuli (Smulders, 2017) and chronic stress (Gualtieri et al., 2019), this suggests the birds not making the detour might be more prone to anxiety and fear than birds making the detour. In mice, lower adult neurogenesis in the ventral hippocampus is associated with higher anxiety levels (Anacker et al., 2018). Alternatively, the hypothesis that only the rostral HF is involved in spatial cognition is too extreme, and spatial information processing happens in the caudal HF as well in birds. Either way, it is consistent with previous work on rodents showing that exposure to chronic stress impaired cognitive abilities (Krugers et al., 1997; Eiland and

McEwen, 2012). However, the results from the analysis linking the hippocampal plasticity analysis to the distance walked during the open field test showed that hens walking less had a lower multipolar DCX+ cell density in the caudal HF than those walking more, which suggests the differences are more likely due to fearfulness.

In addition, the analysis studying the relationship between the distance walked in the arena and the detour performance showed that hens not making the detour walked less than the ones making the detour. This result suggests that hens not making the detour might be more fearful than the ones making the detour. Alternatively, this could be due to differences in coping style, with hens walking more displaying a more proactive behaviour than the one walking less. In addition, the fact that including distance walked in the open field test reduced the significance of the effect of rearing on detour performance indicates that distance walked and rearing share information, with rearing impacting fearfulness even though rearing did not significantly influence distance walked when this test was analysed separately (Dumontier et al., 2022). The early environment thus seems to influence the hens throughout life, up until the end of the production period, in subtle ways that can impact their emotionality and cognition.

5 Conclusions

In conclusion, the rearing environment complexity had long term effects on the hens' ability to master a detour test. This highlights the importance of providing proper housing to chicks, as effects on spatial cognition can last up to the end of the production period. Regarding hippocampal plasticity, the environmental complexity had limited effects on cell proliferation and survival. However, hippocampal plasticity related to the performances in the detour test, with birds performing the detour having a higher DCX+ multipolar cell density in the caudal HF than birds not performing the detour. Results from this study also highlight the role of underlying fearfulness in cognitive testing outcomes and shows just how subtle and complex the effects of early experience can be on the adult phenotype.

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8 Author contributions

LD, JN and AMJ designed and performed the study. LD and JN analysed the data. LD, RLR and TS conducted the hippocampal plasticity part of the study. LD drafted the manuscript and made the figures. All authors reviewed the manuscript and approved the final version.

9 Competing interests

The authors declare no competing interests.

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Paper IV

The effect of rearing and adult environment on HPA-axis responsivity and plumage condition in laying hens

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Abstract

The hypothalamic-pituitary-adrenal (HPA) axis responsivity is influenced by early life experience, but also modified by the environment an individual experiences as an adult. Because laying hens are transferred from rearing to laying farms at 16-18 weeks of age, they are well suited to study the effect of the interaction between early and adult environments on physiology and behaviour. In the European Union, there is a move towards cage-free systems for laying hens, but globally, the majority of layers are kept in cages. Cages have little enrichment and limit the movement of birds and the expression of highly motivated behaviour. Lack of enrichment may lead to under-stimulated birds that are more sensitive to stress and fear-inducing challenges later in life. Fearfulness has been linked to feather pecking, which has negative consequences for animal welfare and productivity. Previous research has shown that birds reared in cages are more fearful and perform more poorly in a test of spatial cognition than birds reared in aviaries when tested within five weeks after transition to adult housing. However, recent results suggest these effects might not be long-lasting. We therefore tested the effect of the rearing (aviary vs cage) and adult environments (standard vs additionally enriched furnished cages) on the corticosterone response to restraint in birds that were 35 weeks into lay and assessed their plumage condition. We hypothesized that lower levels of enrichment, both during rearing and adulthood, would represent a lower level of stimulation, resulting in a stronger corticosterone response to restraint and poorer feather cover. Both stressed (restrained) and control birds increased their corticosterone levels from the first (baseline) to second sample ($p < 0.01$ for both), but the increase in the stress group was significantly higher than in the control group ($F_{1, 111} = 9.51$; $p = 0.003$). There was no effect of the rearing environment, but birds housed in standard furnished cages during lay had overall higher corticosterone levels than birds housed in enriched furnished cages ($F_{1, 51} = 4.12$; $p = 0.048$). Neither rearing nor adult housing influenced feather score except for on the belly, where birds housed in enriched cages had poorer feather score, contrary to our prediction. In conclusion, no effect of the rearing environment on HPA axis responsivity to an acute stressor could be detected 35 weeks into lay, but adult enrichment had a favourable effect on overall corticosterone levels.

Keywords

Poultry, rearing, enrichment, stress, corticosterone, feather quality

1. Introduction

Early life conditions can have life-long effects on the individual. Laying hens are moved from the rearing to the laying farm at 16 weeks of age and this change of environment makes them well suited for investigating effects of early life on the adult phenotype. Early environmental enrichment can increase robustness (Campderrich et al., 2019), while housing in barren conditions may increase fearfulness and decrease cognitive abilities (Brantsaeter et al., 2016ab; Tahamtani et al., 2015; Nazar et al., 2022). Furthermore, individuals reared in a complex environment during the early stages of life may be more frustrated by exposure to a barren environment as adults compared to individuals reared in a barren environment (Luo et al., 2020).

Globally, the majority of poultry are kept in cages (Schuck-Paim et al., 2021). Cages are relatively barren as they give little opportunity to move vertically, to dustbathe and to regulate social encounters. A recent report from the European Food Safety Authority (EFSA) recommends discontinuing the use of cages (EFSA AHAW Panel 2023). Aviary rearing, on the other hand, trains the pullets to navigate vertically, gives more opportunities to perform natural behaviours and avoid aggressive or dominant conspecifics. The variability of the physical and social environment also means birds are intermittently exposed to mild stressors for short durations with the possibility of showing appropriate coping responses. In contrast, life in a barren environment gives little possibility to 'practice' responding to mild stressors and novelty, and this may result in long-term increased sensitivity to stress and increased fearfulness. Fearfulness has been associated with feather damage (de Haas 2014; de Haas et al., 2014), and severe feather pecking, when a bird pecks at and pulls out feathers of a conspecific, is a serious welfare concern for laying hens. Its development may be influenced by the early environment (ibid). Comparison of birds reared in cages and aviaries indicates that birds reared in cages are more fearful, show less active avoidance behaviour

(Brantsaeter et al., 2016ab) and demonstrate poorer cognitive abilities compared to aviary-reared birds (Tahamtani et al., 2015). Laying hens that had been subjected to stress early in life showed a stronger corticosterone response to restraint than control birds at 27 weeks of age (Ericsson et al., 2016). However, when birds were tested at 60 weeks of age, the adult, but not the rearing environment influenced behaviour (Dumontier et al., 2022).

Being housed in a barren environment may be perceived as stressful, even though it is most likely less intense than the stress induced by traditional chronic stress protocols (Rich et al., 2005; Campderrich et al., 2019; Gualtieri et al., 2019). If birds do experience chronic stress, it can have a negative effect both on mental and physical health (Armstrong et al., 2020; Eliwa et al., 2021). Physiological effects of chronic stress include negative effects on adult hippocampal neurogenesis (AHN), the immune system, HPA axis functioning and energy metabolism (summarised in Ostrander et al, 2009; Eliwa et al., 2021), which may reduce the ability of the hippocampus to regulate the stress response (Herman et al., 1989; Herman et al., 1995). An increased corticosterone response to acute stress, and a slightly elevated baseline would thus be expected, but findings include increased, unchanged and reduced baseline and post-stress hormone levels (Gamallo et al., 1986; Marin et al., 2007; Li et al., 2015; Rich and Romero 2005; Gualtieri et al., 2019). Environmental enrichment reduces the consequences of stress and fear (Roy et al., 2001; Morley-Fletcher et al., 2003; Fox et al., 2006; Campderrich et al., 2019). The effect of past and present enrichment may thus interact to influence the phenotype, and the relative importance of each may change over time. To our knowledge, long term effects of the rearing- and adult environment on HPA axis responsivity and plumage condition have not been studied in laying hens.

We therefore tested the effect of different levels of enrichment, past (during rearing) and present (during the production phase), and their interaction, on the physiological stress response and feather cover. The latter was used as a behaviour-related morphological indicator of stress. We predicted that birds exposed to cage rearing and adult housing without additional enrichment would have a stronger corticosterone response to restraint and a poorer feather cover than birds reared in an aviary and housed with additional enrichment

as adults. We predicted that at 52 weeks of age, the production environment used for adult hens would have a stronger impact than the rearing conditions.

2. Materials and methods

2.1 Animal housing and husbandry

2.1.1 Rearing phase

The hens used in this study (N = 256) were part of a larger project for which 384 non-beak trimmed White Leghorn hens were reared either in a cage (N = 192) or in an aviary (N = 192) until they were 18 weeks of age. The birds were reared at a commercial hatchery (Steinlands & co.) in one single room measuring 15 m x 72 m (see Dumontier et al., 2022 for a more detailed description). The system consisted of furnished cages measuring 12 m x 0.8 m x 0.6 m (length x height x width) stacked in three tiers. After hatching, chicks were placed on the first and second tier of the system. The mesh floor of the aviary rows was lined with paper until four weeks of age. From 5 weeks of age, the front of the aviary rows was opened, and the birds could navigate between the different tiers and the floor of the house. The floor of the house was covered with wood shavings. For one of the aviary rows, the front of one tier was kept closed during the whole rearing period. This enclosed space was located in the second tier of the aviary row and contained 250 birds. Thus, they had no access to the floor of the house or the other tiers of the aviary, and this constituted the cage-rearing treatment. From 5 weeks of age, the density was 26 birds/m^2 for the cage-reared birds and 29 birds/m^2 for the aviary-reared birds. In the cage and aviary conditions, birds respectively had access to 9.6 cm and 3.2 cm of perch space per bird.

All birds were exposed to the same lighting and feeding schedule. Temperature started at 34°C and was gradually decreased to 19°C at 16 weeks of age. Birds were exposed to 24 hours of light for the first day, followed by a continuous 4:2 light/dark cycle during the first week as recommended by the Lohman LSL management guide. The light schedule was then switched to 16:8 light/dark at two weeks of age and

gradually decreased to 9:15 light/dark by 5 weeks of age. Gradual transitions from dark to light and from light to dark were used. Each transition took 20 minutes.

2.1.2. Production phase

At 18 weeks of age, the birds were transported to the experimental farm. The henhouse contained 2808 cages organised in 12 rows, each row containing six tiers. A walkway between the 3rd and 4th tier formed the second floor in the henhouse. Experimental birds were all housed in the third tier of the second floor, i.e., the top tier. They were housed in social groups of four individuals in two Victorsson T10 furnished cages connected by an opening (15 cm x 18 cm). The opening between the two cages allowed the birds to move freely between the two cages of the cage-pair. Each pair of cages containing four birds is hereafter referred to as a cage. Each cage measured 240 cm x 83 cm x 63 cm (width x height x depth) and the four birds sharing a cage came from the same rearing treatment. Each cage was furnished with four perches (75 cm perch space / bird), two nest boxes (1500 cm^2 each) and a dustbathing platform on the roof of each nest box (750 cm^2 / bird, Fig. 2). The treatments were distributed in the henhouse so that cages with birds reared in the aviary were next to cages with birds reared in cages.

All birds were exposed to the same lighting and feeding schedule during their time at the farm. From the age of 18 weeks, they were kept under a 13:11 light/dark cycle and a temperature of 21.1±1.6°C without exposure to additional daylight from the outside. Gradual transitions from dark to light and from light to dark were used. Each transition took 15 minutes. Food and water were provided ad libitum via a food chain running in front of the cages and a water line with nipple drinkers along the back of the cages. For identification purposes, each bird was individually marked by means of a black or white plastic zip-tie around its left or right leg.

The additionally enriched cages that made up the enriched treatment of the adult birds were the same as standard control cages with the addition of an extra dustbathing tray for stimulating foraging and dustbathing, a hemp pompon to peck at, and polyethylene tarp curtains to increase structural complexity.

145 The latter were hung under one of the perches of the cage. In addition, a low-density polyethylene (LDPE)
146 sheet was hung on the upper edge of each opening between the two cage halves. Birds could therefore not
147 see past these barriers and either had to move under or around them or push them out of the way to move
148 past them. The extra dustbathing tray (55 cm × 60 cm width × depth with a 2 cm high frame to keep
149 dustbathing material from falling off the tray) was placed on the perches in one half of the cage and refilled
150 weekly with a mixture of feed crumbles and dustbathing pellets made of pelleted wheat husks. The pompon
151 was attached to the cage front above the dustbathing platform so that it hung at the upper half of the cage
152 wall. The enrichment was added to the cages in stages, starting with the platform and polyethylene curtains
153 two weeks after arrival, and then the LDPE sheets and pompons at 26 weeks of age (i.e., eight weeks after
154 arrival).

155 *2.2. Data collection*

156 *2.2.1. Feather score*

157 The plumage condition was evaluated as feather score according to the Welfare Quality® protocol (2009)
158 on all birds in each cage (n=256) at 52 weeks of age. The belly, neck and back were scored for feather cover
159 and given a score of 0, 1 or 2 with 2 being the poorest score.

160 *2.2.2. The restraint stress test*

161 The restraint stress test (Wingfield 1994) was carried out in September 2020. The birds were then 52 weeks
162 of age and had lived at the laying farm for 35 weeks. The enriched treatment group had thus been subjected
163 to their treatment for approximately eight months. One control and one stress bird were randomly chosen
164 from each cage. For half of the cages, the stress bird was sampled first, and for the other half the control
165 bird was sampled first, balanced across rearing and adult treatment. A baseline blood sample was taken
166 from the brachial vein of both birds. The time from opening the cage until securing the blood sample was
167 registered for both birds and used as a covariate in the statistical analysis The control bird was then released
168 back into the cage whereas the stress bird was restrained in a mesh cloth washing bag suspended from a

scaffold for ten minutes. After the ten minutes had elapsed, the stress bird was released from restraint, and a second blood sample was taken from both stress and control. All blood samples were taken by puncturing the brachial vein with a red cannula and collecting the blood with a heparin-coated microvette (Sarstedt). The samples were centrifuged at 2000 g for 5 minutes, and plasma was pipetted off into Eppendorf tubes and immediately frozen on dry ice.

Due to technical difficulties, the final sample size for corticosterone analysis was 232 samples from 118 birds. For four of these 118 birds, only one blood sample was taken, whereas the remaining 114 birds had both the before and after sample analysed (details see Table 1). Only birds from which we got both samples were included in statistical analysis and in figure 2.

Table 1: The tables give an overview over sample size, number of samples and the number of birds for which only one sample was secured for the control and the stress group. The n is the sample size with a complete set of blood samples.

Control birds		
Rearing	Adult housing	
	Enriched	Standard
Aviary	32 (n = 16)	26 (n = 13)
Cage	31 (n = 15; 1 bird one value, otherwise all birds two samples)	30 (n = 15)

Stress birds		
Rearing	Adult housing	
	Enriched	Standard

Aviary	27 (n = 13, 1 bird with one value)	30 (n = 16)
Cage	24 (n = 12)	30 (n = 14; 2 birds with one sample, otherwise all birds two samples)

182

183 *2.3. Corticosterone analysis*

184 Half a millilitre of plasma was extracted with 5 ml diethylether, shaken, centrifuged and frozen. Afterwards
185 the ether phase was transferred into a new glass vial, dried down and re-dissolved in a similar amount of
186 EIA buffer. An aliquot of 50 µl of these extracts was analysed in an in-house corticosterone EIA (for details
187 see Palme and Mostl, 1997).

188 *2.4. Data handling and statistical analysis*

189 All statistical analysis were performed with R, version 4.2.1 (R Core Team, 2022).

190 *2.4.1. Feather score*

191 The feather scores for each of the three areas were analysed in separate models. For the feather score on the
192 neck and back, ordinal logistic regression models (clmm from the ordinal package) with cage included as
193 a random effect and rearing and adult environment and their interaction as fixed effects were applied. P-
194 values were calculated by likelihood ratio tests using the Anova() function from the RVAideMemoire
195 package (Hervé, 2022). For the belly score, the proportional odds assumption did not hold, and for the
196 feather score for this area, a sum of the individual feather scores for each cage was calculated per cage, and
197 the data analysed with a linear model with rearing and adult environment and their interaction as fixed
198 effects.

199

200 *2.4.2. Corticosterone levels*

201 For the corticosterone levels, we used a linear mixed-effects model (LMMs) fitted by restricted maximum
202 likelihood estimates using the lmer function from the R package lme4 (Bates et al., 2015). The model was
203 checked for assumptions (homogeneity of variances and normal distribution of residuals) and the
204 corticosterone concentration had to be log transformed to fit the assumptions. The categorical predictors
205 included in the model were hen ID nested within the cage and the plate ID (used for analysis of
206 corticosterone) as random effects, and rearing (cage or aviary), adult environment (enriched or standard
207 adult housing), treatment (stress or control), and time (first and second sample) as fixed effects. All two-
208 way interactions were included in the model. Post comparisons were done using t-tests. Because
209 corticosterone levels are known to start rising after 3 min from exposure to handling, we checked possible
210 effects of the time to take the first blood sample counted from the moment we opened the cage door to take
211 the first bird out for sampling on corticosterone level. A simple regression model with baseline
212 corticosterone regressed on time to first sample revealed a significant and positive relationship ($F_{1, 114}$ =
213 5.64; p = 0.02), therefore, this covariate was kept in the final model even though it then only tended to be
214 significant.

215 *2.5. Ethics statement*

216 The animals used in this study were enrolled in a larger project. An application for permission to perform
217 the animal studies was submitted to and approved by the Norwegian Food Safety Authority (FOTS ID
218 22443). The experiments were performed in a farm approved as an experimental facility, and the
219 experimental hens were housed in compliance with the Norwegian legislation regarding the use of animals
220 in research (Forskrift om bruk av dyr i forsøk).

221

3. Results

3.1. Feather score

Figure 1 gives an overview over the distribution of the three different feather scores for each of the areas head, neck and belly.

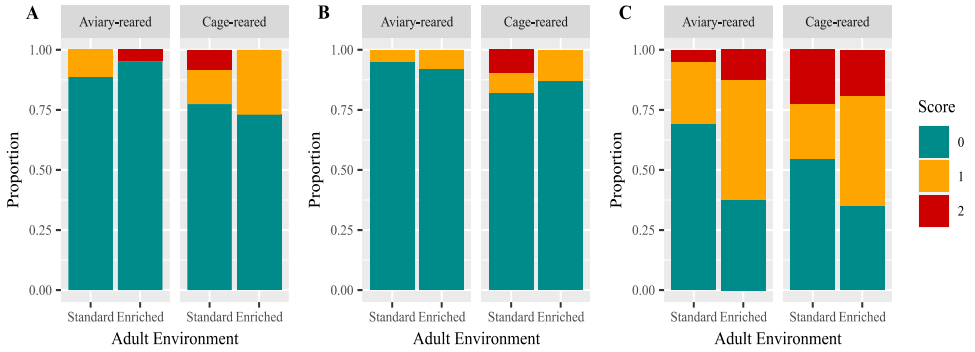

Figure 1: The feather quality score for the neck (A), back (B) and belly (C). Scores range from zero (green bars) to two (red bars), with zero indicating an almost intact plumage and two a very poor condition.

For the neck, neither the provision of enrichment nor the rearing environment affected the feather quality (enrichment: X^2 (df = 1, N = 251) = 0.65; p = 0.42 ; rearing: X^2 (df = 1, N = 251) = 1.56; p = 0.21). The interaction between them was also not significant (X^2 (df = 1, N = 251) = 0.42; p = 0.52).). The same was observed for the back (enrichment: X^2 (df = 1, N = 251) = 0.005; p = 0.94 ; rearing: X^2 (df = 1, N = 251) = 0.59; p = 0.44 ; interaction: X^2 (df = 1, N = 251) = 0.04; p = 0.83). The feather score for the belly was also not influenced by the interaction of rearing and adult environment ($F_{1, 60}$ = 0.82; p=0.37) nor by rearing ($F_{1,60}$ = 2.3; p=0.14). The presence of enrichment in the experimental cages did however increase the belly feather score ($F_{1, 60}$ = 6.1; p=0.016), with enriched birds having an average sum of 7.2 (std 2.2) which was higher than the average sum of 5.9 (std 2.0) for birds housed in standard cages.

3.2. Corticosterone levels

Birds housed in additionally enriched cages during the production period had lower corticosterone concentrations than birds housed in standard furnished cages ($F_{1, 51}$ = 4.12; p = 0.048). However, the interaction between adult housing and stress treatment was not significant. Stressed birds had significantly higher corticosterone levels compared to control birds in the second plasma sample (post- t-test; t_{132}=2.53; p = 0.01), but not in the baseline (p > 0.05; F (sample time*treatment group)$_{1, 111}$ = 9.51; p = 0.003; figure 2)). Both groups increased significantly from baseline (control group: t_{110}=6.93; p < 0.0001 and stress group: t_{111}=10.99; p < 0.0001 for the paired comparison of pre- and post-test sample within group; F (sample time)1, 111 = 162.18; p < 0.0001).

Time-to-first sample tended to have an effect on corticosterone concentration ($F_{1, 71}$ = 3.19; p = 0.08). The corticosterone concentration (ng/ml) is shown for each timepoint and treatment group in figure 2.

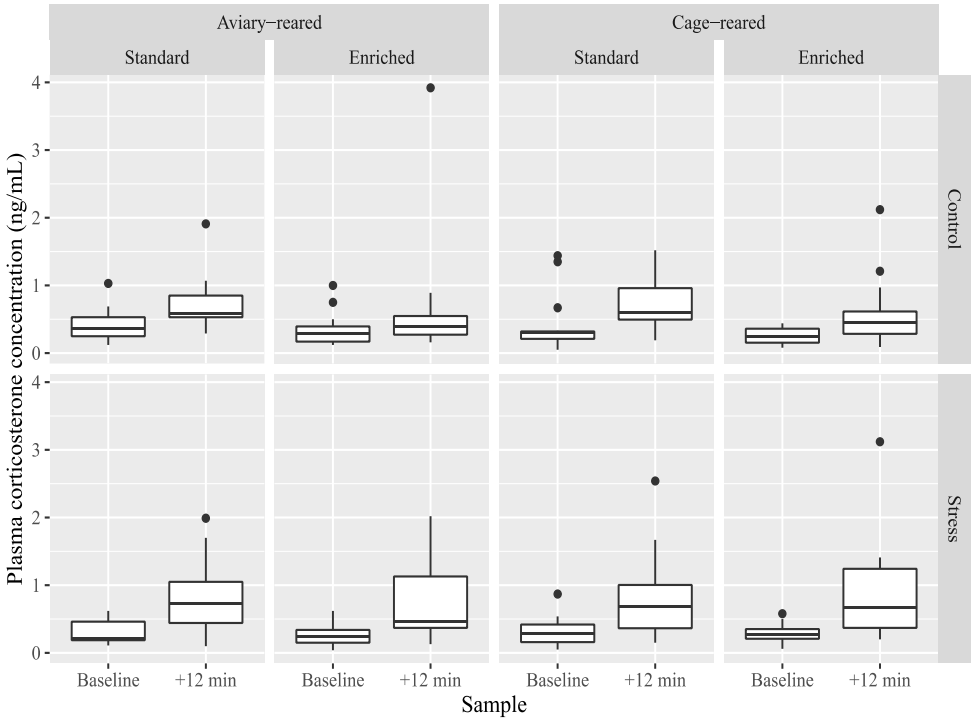

Figure 2: Boxplots of plasma corticosterone concentrations (ng/ml) in control and stressed birds at the first (baseline) and second (+ 12 min) sample for birds reared in an aviary or in cages and housed in enriched or standard conditions during lay.

4. Discussion

The current study was designed to increase our understanding of effects of the early environment on the phenotype of adult laying hens. To this end we tested the effect of cage vs aviary rearing and of adult housing with or without additional enrichment on baseline corticosterone, on the corticosterone response to immobilization and on feather cover as a morphological indicator of stress. We predicted that birds exposed to cage rearing and adult housing without additional enrichment would have a stronger corticosterone response to restraint and a poorer feather cover than birds reared in an aviary with additional enrichment in the home cages as adults. We found no difference in the corticosterone response between birds from the two rearing treatments, and conclude that so far into lay, the rearing environment has no detectable influence on baseline HPA axis activity or HPA axis responsivity after exposure to an acute stressor. Neither was there any interaction between the rearing and adult environment, indicating that birds reared in an aviary and kept in standard furnished cages as adults did not have a stronger sensitivity to acute stress than birds reared in an aviary and provided with enrichment as adults. However, when sample time was accounted for, birds housed with enrichment as adults had lower corticosterone levels. We also found that the procedure of blood sampling and handling alone led to an increased corticosterone secretion, but that the restraint stress had a significantly stronger effect than handling, reflected in a higher corticosterone increase from the first to the second sample in the stress group compared to the control group. Feather quality was influenced only by the adult environment for one of the body areas scored, namely the belly.

The physical restraint stress test (Wingfield, 1994) can be used to measure both response to acute stress and stress recovery depending on the number and timing of blood samples taken. We sampled at baseline and then after twelve minutes, and thus got only the approximate peak of the stress response. The corticosterone levels start to increase after about three minutes after the beginning of stress exposure (Wingfield and Romero, 2001), and ideally, the baseline sample should, therefore, be taken within that time. We did sample each bird within three minutes of capture, but our results show that the birds' stress response was initiated by the first opening of the cage and capturing of the first bird to be sampled. As we sampled one stress and one control bird per cage, we could not sample both birds within three minutes of opening the cage door and controlled for this by including time from the opening the door to blood sample in our statistical analysis, and also by balancing which bird was sampled first across rearing and adult housing treatment and between the stress and control group. The stronger response in the stress group compared to the control group confirms that the restraint test was indeed stressful above and beyond being handled and subjected to blood sampling. Thirty-five weeks into lay, when the hens were 52-54 weeks old, no rearing effects on corticosterone levels or feather quality could be detected. As enrichment in the laying farm but not the rearing environment had an effect on overall corticosterone levels, it seems that in adult birds, the production environment has a stronger impact on HPA axis activity than the early rearing environment. This aligns with the results reported in Dumontier et al. (2022), where adult enrichment, but not rearing conditions influenced the birds' response to a novel object at 60 weeks of age. However, the interpretation of the biological importance of the administered enrichment for the stress response is more difficult, as the effect of enrichment was significant only when sample time (before and after stressor) had been accounted for and did not differ between the stress and the control group.

Ericsson and colleagues (2016) tested hens in the restraint stress test at 29 weeks of age, following stress administered at two, eight and 17 weeks of age, and found a higher corticosterone response to restraint in the birds that had been stressed at eight weeks of age. As in our study, baseline corticosterone was not influenced. However, the administration of stressors over short periods of time is not directly comparable

to housing in cages. Studies of housing effects on corticosterone levels show an increase in feather corticosterone in hens housed in conventional cages compared to hens housed in floor pens (Campbell et al., 2022). Therefore, measures of HPA activity which reflect cumulative effects of longer periods of exposure to different environments could have been a better way of assessing effects of the rearing treatments and adult housing on baseline corticosterone levels.

The enrichment we administered consisted of a dust-bathing platform made out of wood, with elevated edges to keep the dustbathing material on the platform. We provided birds with enough dustbathing material to cover the platform and refilled it once every week. Hens are highly motivated for dustbathing, and they will work to obtain access to it, and increase their dustbathing frequency when given access if access has previously been denied (Widowski and Duncan, 2000). Interestingly, Barnett et al. 2009 found no effect of giving access to a dustbathing platform for 6 h a day on plasma corticosterone or on the response to an ACTH injection, even though the platform was frequently used.

The feather quality on different body areas is believed to indicate different welfare challenges: Aggressive pecking centres around the head, feather pecking around the tail, and vent pecking is close to the vent (see the Welfare Quality protocol and figure 1 in de Haas et al., 2021). The feather cover on the belly is not that easily interpreted. It is suggested in the Welfare Quality protocol that it is influenced by production and that it can be reduced in highly productive birds. We did no measure egg production, but we have no reason to believe that there were large production differences between hens housed with and without extra enrichment. We did not observe any skin wounds or vent pecking, so the feather loss on the belly did not seem to be caused by injurious pecking. Our impression from observing the hens was that they used the platforms very frequently, often dustbathing all four hens together, in line with Duncan's description of dustbathing as a socially facilitated activity (Duncan et al., 1998). This was however not quantified. It is possible that feathers were worn away by frequent use of the wooden shelf for dust bathing. Importantly,

feather cover on the neck and back was not influenced by any of our treatments. This suggests there was no difference in severe feather pecking between the rearing and adult housing treatments.

5. Conclusion

In conclusion, when hens were reared in either an aviary or in cages, and then housed in standard Victorsson T10 furnished cages or additionally enriched cages as adults, neither rearing nor the interaction between the rearing and adult environment influenced baseline corticosterone or the acute hormonal stress response. Additional enrichment in adult housing conditions lowered the overall corticosterone levels. The enriched birds had a poorer feather cover on their bellies, but this is probably related to the physical characteristics of the dustbathing shelves, and was probably not a consequence of access to the enrichment per se.

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