Early environmental effects on laying hen development

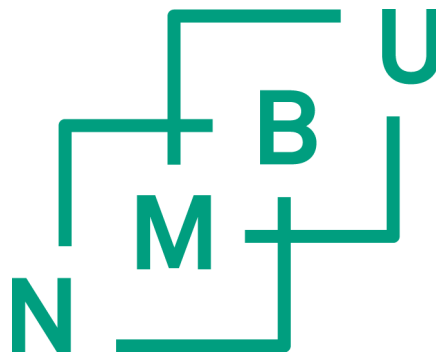Philosophiae Doctor (PhD) Thesis

Fernanda Machado Tahamtani

Department of Production Animal Clinical Sciences

Faculty of Veterinary Medicine and Biosciences

Norwegian University of Life Sciences

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Summary

Chickens are widely used by humans in both farm production and in scientific research as model organisms. In the farming industry, they total 27 billion individuals worldwide (FAO, 2013). Over 6 billion of these are laying hens in egg farms (FAO, 2013). The large majority of these animals are reared and housed in cages (Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015). However, recent governmental and scientific concern for farm animal welfare has resulted in a growing number of countries and regions banning the use of conventional cages and adopting other housing systems, such as furnished cages, aviaries, and free-range systems. These systems provide quite different and more complex environments for the laying hens than conventional cages. It is known that the environment is an important factor in the behavioural and physiological development of animals. Therefore, the different rearing and housing systems for laying hens may result in differences in the behavioural development of these birds. This would be of particular importance in the event of the rearing and housing environments experienced by the birds being different. In Norway, due to national and European legislation, laying hens are largely reared in aviaries and may be transferred to production farms that use furnished cages. There is a worry that these individuals might suffer frustration and stress due to environmental restriction compared to hens that were reared in cages and are, perhaps, adapted to such an environment.

The aviary and the cage housing systems for laying hens can be very different, particularly in complexity. The aviary environment provides larger three-dimensional space, allowing the expression of natural behaviours such as wing flapping and flying. Furthermore, in the aviary environment, resources such as food, water, nest boxes, and perches are distributed in this large space, and the hens must find and be able to access them. On the other hand, the cage environment provides all resources to the birds in a much smaller space, removing any necessity to search for these. These environmental distinctions between aviary and cage systems arguably require different levels of spatial cognitive skills. As brain and nerve tissues are energetically costly to develop and maintain (Armstrong, 1983; Isler and Van Schaik, 2009; Niemela et al., 2013), it might be expected that the hens will allocate resources in such a way as to maximise production (Schutz et al., 2002) and therefore only possess specialized spatial cognition skills if they are necessary.

Intensive farming often causes conditions or disorders that reduce the welfare of the farmed animals. In the egg farming industry, one of these main issues is that of feather pecking, the forceful pulling and removal of feathers of conspecifics (Savory, 1995). Feather pecking is largely attributed to the improper provision of litter, causing redirected pecking (Blokhuis, 1986; Newberry et al., 2007). Indeed, a recent observational study has suggested that litter as simple as a layer of paper on which dust and droppings can accumulate, may be enough to reduce the incidence of feather pecking (de Haas et al., 2014b). However, no experimental work has tested the effects of the provision of paper on the development of feather pecking in the full-scale, real-life context of egg farming.

The work presented in this thesis set out to fill some of the knowledge gaps described above. Paper **I** aimed at comparing welfare and productivity in aviary- and cage-reared hens housed in a furnished cage production farm. Papers **II** and **III** aimed at testing whether rearing in aviary or cage systems influences spatial memory and whether these different methods of rearing cause differences in tyrosine hydroxylase in the hippocampus and the caudolateral nidopallium, respectively. Paper **IV** aimed at testing the effects of providing paper substrate from the first day of life on the incidence of feather pecking and feather damage at the peak of lay. The results from paper **I** showed that aviary-reared hens demonstrate indicators of better welfare in the first three weeks after transfer to a furnished cages production system compared to cage-reared hens. However, over the course of the whole production period, mortality of aviary-reared birds housed in furnished cages was higher than the mortality of cage-reared birds housed in furnished cages. In paper **II**, it became apparent that aviary rearing promotes spatial memory development whereas cage-rearing causes long-term impairment of short-term memory, as seen by the superior working memory performance of aviary-reared birds in a holeboard task, compared to cage-reared birds. Following the results from paper **II**, paper **III** was devised to investigate whether aviary vs. cage rearing results in differences in the dopaminergic pathway, specifically tyrosine hydroxylase, in the areas of the brain responsible for cognitive and executive functions. The results did not show any treatment effects on tyrosine hydroxylase in the hippocampus or the caudolateral nidopallium. In paper **IV**, it was found that hens provided with paper had a lower probability of having feather damage compared to hens that did not have access to paper. In addition, the results showed that

production farms that provided environmental enrichment had a lower incidence of gentle feather pecking.

These results, seen in the context of existing literature, suggest that rearing in complex environments produces hens that are more resilient and better capable of coping with environmental changes, at least in the short term. It is demonstrated that the environment experienced during rearing can have considerable, both short- and long-lasting, effects on the behavioural development of laying hens and on how well they will cope with being housed in the planned adult environment. This, in turn, carries pertinent welfare consequences.

Sammendrag

Verpehøner og slaktekyllinger benyttes i stort omfang av mennesker både i landbrukssammenheng og som modellorganisme innen forskning. På verdensbasis er det totale antallet individer slaktekylling og verpehøner innen landbruket 27 milliarder dyr. Over 6 milliarder av disse er verpehøner. Majoriteten av dyrene holdes i bur under oppdrett og eggproduksjon (Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015). I nyere tid har blitt mer fokus på dyrevelferd blant politikere og forskningsmiljø, og dette har resultert i at stadig flere land forbyr bruk av tradisjonelle 3-hønersbur. Dette har ført til en endring i oppstalling av dyrene som promoterer miljøbur og frittgående systemer (aviarier) med eller uten tilgang på uteareal. Disse oppstallingssystemene utsetter hønene for forskjellige miljø, særlig med hensyn på kompleksitet. Da miljøet dyrene lever i er viktig for dyrenes fysiologiske og mentale utvikling, kan oppstalling under både oppdrett og produksjon påvirke dyrenes utvikling. Dersom dyrene oppdrettes i en type miljø, for så å bli flyttet til et annet miljø for eggproduksjon, vil dette kunne påvirke dyret negativt. I Norge reguleres hold av fjørfe via regler diktert av EU, samt nasjonalt regelverk. I Norge oppdrettes de fleste høner i aviarier, men kan bli sendt enten til miljøbur eller til aviarier for eggproduksjon. For høner som er vant til å være i et miljø hvor de har stor bevegelsesfrihet og gode muligheter til å utøve naturlig atferd (aviarier), kan overgangen til oppstalling i et mer restriktivt miljø (innredede bur) medføre frustrasjon og stress. For høner som derimot er oppdrettet i bur, vil trolig overgangen til bur ikke medføre like stor påkjenning.

Aviarier og bur skiller seg ut på ulike måter, men særlig hva angår kompleksitet. I et aviar kan dyrene bevege seg både i ulike høyder samt langs bakken. Dyrene kan derfor få utført viktige atferder som å fly og å flakse med vingene uten å støte borti innredning eller andre høner. I aviarier er mat, vann, redekasser og vaglepinner spredt utover et større område og det krever mer av hønene for å klare å finne fram til, og benytte seg av tilgjengelig ressurser. I bur derimot har dyrene tilgang på alle ressurser innenfor et mindre område, noe som medfører mindre utfordring med å lokalisere og finne mat, vann, vaglepinner og redekasser. Forskjellene disse ulike oppstallingssystemene har, medfører sannsynligvis ulike behov for kognitive egenskaper og romlig forståelse. Det er kostbart for dyret å utvikle og opprettholde nevrologiske nervebaner (Isler and Van Schaik, 2009; Niemela et al., 2013). Det forventes at hønene allokerer energien for å maksimere produksjon (Schutz et

al., 2002) og at de som ikke har behov for å bruke spesielle evner heller ikke vil allokere energi til å utvikle dem.

Kommersielt dyrehold innebærer ofte at dyrene holdes under forhold som kompromittere og reduserer dyrevelferden. I eggproduksjonen er fjørhakking et av hovedproblemene man står overfor. Fjørhakking er når høner napper så kraftig i fjør at fjørene løsner og fjernes (Savory, 1995) fra egen eller andres fjørdrakt. Fjørhakking settes i stor grad i sammenheng med mangel på annet hakke-substrat, noe som fører til at hakke-adferden styres mot andre høner heller enn mot substrat på bakken (Blokhuis, 1986; Newberry et al., 2007). En nylig publisert observasjonsstudie tyder på at noe så enkelt som et tynt lag med papir hvor støv og andre partikler kan akkumulere, kan være nok til å redusere fjørhakking (de Haas et al., 2014b). Det er imidlertid ingen eksperimentelle studier som har testet om dette enkle tiltaket fungerer for å motvirke fjørhakking i industriell eggproduksjon.

Arbeidet som presenteres i denne avhandlingen har som mål å svare på noen av de omtalte problemstillingene. Målet med artikkel **I** var å sammenlikne dyrevelferd og produktivitet hos bur- og aviaroppdrettede høner oppstallet i miljøbur hos eggprodusenten. Artikkel **II** og **III** testet om bur- eller aviaroppdrett påvirket romlig forståelse og hukommelse, og om oppdrettsformen forårsaket forskjeller i enzymet tyroksinhydroksilase i hippocampus og *nidopallum caudolaterale*. Artikkel **IV** hadde som mål å teste om tilgang på hakke-substrat fra første levedag påvirket forekomsten av fjørhakking og kvalitet på fjørdrakten når hønene var på verpetopp. Resultatene fra artikkel **I** viste at høner oppdrettet i aviarier utførte mer trivselsadferd de første tre ukene etter overgang til innredede bur sammenliknet med høner som var oppdrettet i bur. Samtidig var dødeligheten for produksjonsperioden høyere blant aviaroppdrettede høner sammenliknet med buroppdrettede høner. I artikkel **II** viste det seg at oppdrett i aviar tilrettela for utvikling av bedre korttidshukommelse. Som en oppfølging av resultatene fra artikkel **I** og **II**, undersøkte artikkel **III** om forskjellen i hukommelse var relatert til forskjeller i dopaminerge nervebaner i hjerneavsnitt som er ansvarlige for kognisjon og beslutningstaking. Resultatet fra artikkel **III** viste at det ikke var forskjell i menge tyrosinhydroksilase, det hastighetsbegrensende enzymet i dannelsen av dopamin, i verken hippocampus eller i *nidopallum caudolaterale* (tilsvarer dorsolateral prefrontal cortex hos pattedyr). Artikkel **IV** demonstrerte at tildeling av papir som substrat reduserte

sannsynligheten for dårlig fjørdrakt sammenliknet med høner som ikke fikk tilgang på hakke-substrat. I tillegg viste resultatene fra artikkel **IV** at tilgang på berikelse hos eggprodusenten reduserte forekomsten av milde former for fjørhakking.

Når resultatene fra dette arbeidet settes i sammenheng med allerede publisert litteratur, kan det konkluderes at oppdrett i mer komplekst miljø produserer høner som er mer robuste og bedre til å tilpasse seg miljøforandringer på kort sikt. Samtidig vektlegges det at miljøet under oppdrettsfasen har både korttids- og langtidseffekter på utvikling av verpehøners atferd, og påvirker hvor egnet de er til å oppstalles i ulike miljøer som voksne. Dette har konsekvenser for dyrevelferd.

List of papers

Paper I

Does rearing laying hens in aviaries adversely affect long-term welfare following transfer to furnished cages?

Tahamtani, F. M., Hansen, T. B., Orritt, R., Nicol, C., Moe, R. O., Janczak, A. M, 2014. PLoS ONE 9(9):e107357. DOI: 10.1371/journal.pone.0107357

Paper II

Early life in a barren environment adversely affects spatial cognition in laying hens (*Gallus gallus domesticus*).

Tahamtani, F. M., Nordgreen, J., Nordquist, R. E., Janczak, A. M., 2015. Frontiers in Veterinary Sciences 2:3. DOI: 10.3389/fvets.2015.00003

Paper III

Does early environmental complexity influence tyrosine hydroxylase in the chicken hippocampus and "prefrontal" caudolateral nidopallium?

Tahamtani, F. M., Nordgreen, J., Brantsæter, M., Østby, G. C., Nordquist, R. E., Janczak, A. M., 2016. Frontiers in Veterinary Sciences 3:8. DOI: 10.3389/fvets.2016.00008

Paper IV

Effects of litter provision during early rearing and environmental enrichment during the production phase on feather pecking and feather damage in laying hens.

Tahamtani, F. M., Brantsæter, M., Nordgreen, J., Sandberg, E., Hansen T.B., Nødtvedt, A., Rodenburg, T.B., Moe, R. O., Janczak, A. M.

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1. Introduction

Chickens (*Gallus gallus domesticus*) are widely used by humans in both farm production and in scientific research as model organisms. In the farming industry, these animals are used for both meat and egg production, totalling approximately 20 billion broiler chickens produced worldwide per year in the meat industry and 6.6 billion hens in the egg industry (FAO, 2013). Thus, the chicken is a highly relevant species for both basic and applied research. The concern for animal welfare, coupled with increasing scientific knowledge of the behavioural and physical needs of farm animals, has been guiding the legislation of several countries on how these animals should be farmed. Conventional cages for laying hens, for example, were banned in Europe due to a combination of the results from behavioural research, showing that hens are motivated to dust-bathe, perch, and lay their eggs in a nest, and the public concern on how laying hens were housed for farming (see section 1.1.1). In Norway, since the European ban on conventional battery cages for laying hens in 2012 (Council of the European Union, 1999), nearly all laying hens are reared in aviaries and later housed in aviaries or in furnished cages during the laying period (Landbruks- og matdepartmentet, 2001). However, even though both housing systems offer access to perches, dustbathing opportunities and access to a nest box, there are large differences between the aviary environment and the furnished cage environment, particularly in overall complexity. Aviaries offer more three-dimensional space and opportunity for the birds to perform natural behaviours such as flying and dustbathing. Laying hens in aviaries also have contact with a much larger number of conspecifics and thus have more potential for positive and negative social interactions. Therefore, chicks may spend the rearing period, the first 16 weeks of life, in aviaries and be sent to production in furnished cages despite the potential stress caused by transferal from loose house rearing to space-restricted cages. Some farmers worry that the difference in rearing and production environments might reduce the welfare and productivity of the flock. Rearing farmers will often simulate a cage environment in the rearing aviaries when they know these birds will be sent to furnished cage laying systems. This, however, largely alters the environment the hens can experience during rearing. In turn, this alteration in the environment may affect aspects of the behavioural development in the hens (section 1.1).

In this thesis work, we investigated how the rearing environment affects the development of certain behaviours in laying hens and what consequences this may have for their welfare. To this end, we measured outcomes such as comfort- and aversion-related behaviours (section 1.2), feather pecking (section 1.3), and cognitive ability (section 1.4).

1.1 Behavioural development of the chicken

The domestic chicken was domesticated from the wild ancestor, the red jungle fowl (*Gallus gallus*), and several breeds have undergone artificial selection for high productivity. This domestication process incurs relaxation of natural pressures, such as food shortage and predation, as a consequence of life under human supervision (Andersson et al., 2001; Jensen, 2014). This relaxation reduces the fitness benefit of having energetically costly adaptations to pressures that no longer exist (Schutz et al., 2002). For example, if food is provided by humans, the need to spend energy searching for food decreases. However, it has been found that domestication only changed the frequency of the performance of certain behaviours, but not the form or the motivation to perform these behaviours. For example, the red jungle fowl performs exploratory behaviours at a higher frequency than domesticated laying hens (Andersson et al., 2001; Schutz and Jensen, 2001), while a layer breed ingests food at a higher frequency than the red jungle fowl (Andersson et al., 2001). Furthermore, compared to the red jungle fowl, White Leghorn layers show less contrafreeloading, less caution in a test arena, and impaired spatial learning (Lindqvist and Jensen, 2009).

A series of behaviours develops in the chicken in the first few weeks after hatching. While still inside the egg, the chick receives a range of stimuli, such as light, olfactory, and auditory cues, stimulating the development of the respective neurological pathways (reviewed in Rogers, 1995b). Imprinting, the process by which the chick learns to recognise the hen and develops a social preference for it, occurs in the first 48 hours post hatching (Bateson, 1966; Bolhuis, 1991). In the absence of the mother hen or brood mates, studies have shown that the chicks can imprint on a range of animate and inanimate surrogates (Bateson, 1966). Fear behaviours develop at the same time as imprinting takes place, with chicks starting to avoid and show fear responses to novel stimuli (Sluckin and Salzen, 1961). Perching behaviour starts from the second week of age

(Heikkilä et al., 2006) and it has been shown that early life without access to perches impairs spatial cognitive skills of the chicken later in life (Gunnarsson et al., 2000).

Learning to feed takes place during the first two weeks post hatching. Immediately after hatching, the young chick pecks at small spherical, three-dimensional objects (Dawkins, 1968). This early pecking behaviour, however, is done with the beak closed and is investigatory in nature (Rogers, 1995a). The yolk sac can still provide nutrients for the first three days post hatching; therefore, these investigatory pecks have a function in learning to discriminate food from inedible objects (Freeman, 1965; Rogers, 1995a). The colour of objects can also stimulate the newly hatched chick. Chicks more readily peck and approach objects that are red and blue (Clifton and Andrew, 1983). Pecking, with the beak open or closed, promotes tactile feedback, which works to form a reward system (Hogan, 1973). During the first 4 days of life, the chick collects information on the suitability of objects to be pecked and then swallowed (Hogan, 1973). Two-week-old chicks are able to distinguish between feed grains and pebbles within 60 pecks (Reymond and Rogers, 1981). Social facilitation also plays a role in early pecking responses. Food ingestion is greater in the presence of a companion chick, particularly if the companion is also feeding (Tolman and Wilson, 1965; Tolman, 1968). Feeding behaviour is also facilitated by companion and hen models and by tapping sounds (Tolman, 1964; Tolman, 1967b; a). Chicks have been shown to develop pecking target preferences after observing a model pecking at a particular type of target (Turner, 1964). In the first three days post-hatching, chicks are susceptible to this type of social facilitation and the pecking preferences developed then lead to stable food preferences (Suboski and Bartashunas, 1984). While watching a model pecking, the chicks establish a search image and have been shown to peck at objects that share given characteristics, and therefore fit the search image, rather than at random (Andrew and Rogers, 1972). Feather pecking, a common behavioural disorder in laying hens in which individuals pull out feathers from their conspecifics, is largely attributed to the absence of litter, causing the birds to redirect ground pecking to the feathers of their companions (see section 1.3).

1.1.1   Egg farming

As seen in the previous section, it is apparent that the environmental conditions and the experiences young chicks have with their specific environment have effects on aspects of the development of

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their behaviour. In the egg farming industry, several housing systems for laying hens are used. The large majority of laying hens worldwide are reared and housed in cages (Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015). Nevertheless, public interest in the welfare of farmed animals combined with the increased scientific knowledge about behaviour in laying hens has been an incentive for several countries to adopt laws and directives that prioritise animal welfare. Conventional cages were first introduced as a housing system for laying hens for economic reasons and in an effort to control the spread of disease and parasites (Duncan, 2001; Tauson, 2005). The cage has a wire mesh floor, which allows faeces to drop onto a manure belt, keeping the cage environment clean. In addition, housing the birds in smaller groups further limits the spread of damaging pecking (Duncan, 2001; Tauson, 2005). However, since the implementation of conventional cages, the scientific knowledge of laying hen behavioural needs and motivations has increased (see sections 1.2 and 1.2.1). For example, laying hens are motivated to perform behaviours such as dustbathing, wing stretching, wing flapping, and to use a nest box during egg laying (Black and Hughes, 1974; Duncan and Kite, 1989). The conventional cage environment, however, does not allow the performance of these behaviours, resulting in potential suffering from deprivation (Dawkins, 1988; Tauson, 2002). This increase in scientific knowledge, therefore, led to the European Union banning the use of conventional cages for laying hens in 2012 (Council of the European Union, 1999). Also in 2012, New Zealand passed a code of welfare to guide poultry farmers on the minimum standards of housing and husbandry (Ministry for Primary Industries, 2013). In 2015, the state of California, USA, banned the sale of eggs produced by hens housed in conventional cages (Huffman, 2010). In addition to the legislation, some countries, the UK, for example, have a range of Quality Assurance Standard labels that guarantee the minimum requirements and often ensure higher standards of welfare (Compassion in World Farming, 2016).

With the ban on conventional cages for laying hens, furnished cages were introduced as an alternative (Council of the European Union, 1999). They provide more space per hen and allow the hens to perform some of these highly motivated behaviours such as dustbathing, perching and nesting (Council of the European Union, 1999). Furthermore, in furnished cages, the hens are still housed in small groups and on a wire mesh floor, both factors helping prevent the spread of disease and cannibalistic pecking through the flock, and facilitating the establishment of a stable social structure (Duncan, 2001; Appleby et al., 2002; Tauson, 2002; Keeling et al., 2003). The furnished

cages, therefore, were developed with the objective of decreasing the disadvantages of behavioural deprivation of conventional cages while maintaining the economic and hygienic advantages (Tauson, 2002). Nevertheless, the public interest in animal welfare and the increase in scientific knowledge on animal behaviour and health continue causing changes in how laying hens are housed. While countries like Brazil and India still house 100% of their farmed laying hens in cages, conventional or furnished, other countries have a higher percentage of farmed laying hens in aviaries or in free-range systems (Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015). Free-range systems are used to house 14% of layers in South Africa, 28% in Australia, 45% in the UK, and 75% in Switzerland (Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015).

In Norway, the rearing period, which consists of the first 16 weeks of life of the laying hen, is generally spent in an aviary rearing system (Landbruks- og matdepartmentet, 2001). In this system, the chicks arrive at one day of age and must be kept inside the aviary rows where they have access to food and water. They are kept in these rows until they are sufficiently grown, generally at five weeks of age, and can be released onto the aviary floor to take advantage of the space of the rearing house as a whole. Once they reach that age and the aviary rows are opened, the pullets have the opportunity to move in three-dimensional space and to perform a wide range of natural behaviours such as wing flapping, dustbathing, and flying. In addition, they have both positive and negative contact with a large number of conspecifics. In Norway, a typical laying hen aviary-rearing house contains more than 15000 birds (Landbruks- og matdepartmentet, 2001). In the case of negative (antagonistic or aggressive) social interactions, a subordinate chicken has the option of moving away from the area to avoid or escape the attacker (McLean et al., 1986) but is in constant contact with new individuals. The chickens must also be able to find and reach food troughs, drinking nipples, and perches throughout the aviary. At 16 weeks of age, the birds are transported to the production farm, in which they will reach maturity, start laying, and remain there until slaughter at 70-80 weeks of age. In Norway, the production houses are organised either as aviary systems, which are much as the rearing aviaries but with the addition of nest boxes, or as furnished cage systems. Producers using furnished cage systems are concerned that adaptation to the more spatially restrictive environment of the furnished cage after rearing in aviaries may cause welfare problems for the birds. Reduced welfare in this context may result from frustration, the emotional response to thwarting of access to expected resources (Haskell et al., 2000), or stress caused by

exposure to environmental change. These mechanisms are not mutually exclusive and are likely to exacerbate the stress caused by transfer from the rearing to the production farm and other physiological changes associated with the start of lay at approximately 18 weeks of age (Bestman et al., 2011).

A common practice among Norwegian rearing farmers is to simulate the furnished cage environment some birds may experience during the production period. In order to do this, the rearing farmers do not let the chicks out from the aviary rows during the rearing period. The hens are therefore kept inside the cages in the aviary row during the first 16 weeks of life, thus creating a group of "cage-reared" laying hens. This results in the aviary-reared hens and cage-reared hens experiencing largely different rearing environments particularly in regards to environmental complexity. In a cage rearing system, the chickens have very limited space in which to move. All resources available to each hen are within the cage and, therefore, the birds need not search for these. In addition, each hen only has physical contact with 20 or so conspecifics. These differences in environmental complexities potentially have large effects on the behavioural development of these birds. This may, in turn, have effects on how the birds cope with environmental change and stressful conditions.

1.2   Assessment of animal welfare

In 1964, Ruth Harrison published the book "Animal Machines" which drew public attention to how farm animals are housed and treated in industrialised agriculture (Harrison, 1964). As a response to this public interest, in 1965, the UK Ministry of Agriculture held an expert committee to look into the welfare of farm animals. The committee, chaired by Professor Brambell, presented a report entitled "Report of the Technical Committee to Enquire into the Welfare of Animals Kept under Intensive Livestock Husbandry Systems", which became known as the Brambell Report (Brambell Committee, 1965). From this report came one of the first definitions of animal welfare:

"Welfare is a wide term that embraces both the physical and the mental well-being of the animal. Any attempt to evaluate welfare therefore must take into account the scientific evidence available concerning the feelings of animals that can be derived from their structure and functions and also from their behaviour".

More recent work also highlights the importance of how the animals feel, in addition to their physical health, towards the assessment of animal welfare. Webster et al. (2004) defined welfare as the animal being "fit and feeling good". Likewise, Dawkins (2004) proposed that assessment of animal welfare should be performed by answering two questions: (1) Are the animals healthy? (2) Do they have what they want? In addition, Dawkins (2004) suggests that assessing behaviour is a vital part in answering both these questions with the use of, for example, choice and preference tests. With these definitions of animal welfare, it is clear that welfare extends beyond the presence or absence of disease as it includes mental aspects as well as any physical aspects of quality of life (Dawkins, 1990). Certainly, disease and poor health are threats to welfare. Diseases are likely to cause pain and discomfort; they can weaken the individual by reducing its ability to procure resources, such as food and water, and to avoid predators or antagonistic interactions with conspecifics, promoting further injury and distress (Cockram and Hughes, 2011). However, animals may still suffer poor welfare while in good physical health, namely if they are deprived of activities and resources they are highly motivated for (Dawkins, 2004). Here enters the importance of the behavioural assessment of welfare. The work presented in this thesis focused mainly on behavioural indicators of welfare, and therefore, relates more to the mental well-being rather than physical health aspects of welfare. Throughout the discussion of the results in this thesis, the word "welfare" has been used to mean mental well-being specifically. The physical health-related component of welfare is also discussed where appropriate, but it is specified by the use of the words physical health.

1.2.1   Behavioural indicators of welfare

Behavioural assessment is a common tool for ethologists and welfare scientists as it is non-invasive. Behavioural indicators of welfare stem from the behavioural priorities of the animals, those that individuals are highly motivated to perform, and from those that are considered abnormal (Cooper and Albentosa, 2003). In laying hens, the named "comfort" behaviours, such as wing flapping and dustbathing, are those that are associated with positive choice and that decline in frequency when the animal is under stressful conditions (Nicol et al., 2009; Nicol et al., 2011a). Also associated with positive choice are flying, foraging, and alert behaviour towards a novel object (Nicol et al., 2011b). These behaviours serve the purpose of maintaining the hen's mental

and physical wellbeing (de Jong et al., 2007; Moe et al., 2014). On the contrary, aversion or displacement behaviours, such as head shaking, feather pecking, self-scratching, and short bouts of preening, are associated with negative choice and mild stress (Duncan and Wood-Gush, 1972a; Haskell et al., 2000; Taylor et al., 2001; Nicol et al., 2009; Nicol et al., 2011b). Thwarting access to resources or rewards increases frustration-induced aggression and pacing behaviour (Haskell et al., 2000). Frustration due to thwarting also increases the rate of displacement preening or preening bouts of short duration (Duncan and Wood-Gush, 1972a). On the other hand, long bouts of preening or self-grooming have been associated with positive choice in laying hens (Nicol et al., 2009).

Laying hens have high motivation towards dustbathing, will show increased frequency of this behaviour after a period of deprivation, and will even perform vacuum dustbathing, in which dustbathing behaviours are seen in the absence of substrate if deprivation is long enough (Black and Hughes, 1974). Likewise, the performances of wing stretching, feather raising, tail wagging, leg stretching, and wing flapping increase at a "rebound" rate after a long period of space deprivation (Nicol, 1987). These studies show that chickens are highly motivated to perform these behaviours and suggest that depriving these animals of the opportunity to do so possibly induces suffering (Dawkins, 1988). Social factors may also influence the frequency of comfort behaviours, with preening, feather raising, and tail wagging all occurring more often when hens are in close proximity to pen mates (Nicol, 1989). This also supports the notion of comfort behaviours being indicators of a positive mental state as chickens are prey animals that live in large groups and are highly stressed by isolation. On the other hand, high stocking density can inhibit the performance of comfort behaviours and this can be due to a lack of physical space or due to behavioural inhibition (Albentosa and Cooper, 2004). Comfort behaviours also have a higher frequency during anticipation of positive events following exposure to a classically conditioned stimulus compared to a negative or neutral event (Zimmerman et al., 2011).

In 2009, the European Welfare Quality® project developed a standardised method for assessing animal welfare and published the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009). This protocol is intended to be used for on-farm and/or at slaughterhouse assessment and is divided into four sections, each investigating a specific component of welfare:

feeding, housing, health, and behaviour. However, the estimated time required for completing the assessment with the full protocol is over six hours. In addition, it has been suggested that comfort and aversion-related behaviours are somewhat unrelated to other welfare indicators, such as body temperature, heterophil:lymphocyte ratio, and other physiological measures and should, therefore, be an independent measure of welfare (Nicol et al., 2011a). In my thesis work, therefore, this protocol was used as a general guide, using only the behaviour assessment section and adapting the methods to best suit my study and target population. We performed direct behavioural observations of comfort behaviours, aversion-related behaviours, and feather pecking. In addition, we performed an assessment of feather damage as an indirect measure of feather pecking.

1.3 Feather pecking

Feather pecking is one of the major welfare issues faced by the egg farming industry worldwide (EFSA, 2005). It can be divided into several categories, depending on the motivation and target of the pecking bird (Savory, 1995). Aggressive pecking occurs when a dominant individual targets a subordinate individual with the goal of establishing and maintaining dominance. It is generally directed at the head, but can be aimed at other body parts if the head is out of reach. Aggressive pecks are given with considerable force and give the impression that they are intended to hurt. The recipient will often respond by vocalisations and by withdrawing itself from the area, putting some distance between themselves and the pecker. This type of pecking may cause tissue damage, often seen as peck marks on the combs of subordinate individuals. If it is impossible for the subordinate to withdraw and avoid these antagonistic interactions, persistent aggressive pecking may lead to severe injury or death.

Gentle feather pecking causes little to no harm to the tissue (Kjaer and Vestergaard, 1999). This type of pecking is often directed at the tip or edges of tail feathers or at feathers that are dishevelled and, therefore, stand out from the rest of the plumage (McAdie and Keeling, 2000). It can also be directed at dust or food particles lying on the plumage of other birds. It often takes a stereotypic character, occurring in multiple bouts of several sequential pecks (Kjaer and Vestergaard, 1999; McAdie and Keeling, 2002). Gentle feather pecking is, however, mostly ignored by the recipient. On the contrary, severe feather pecking involves the grasping and pulling of feathers, often followed by feather eating. The recipient birds will generally respond with sharp vocalisations and

withdrawal (Savory and Mann, 1997). Severe feather pecking can result in exposing denuded areas of skin that will, in turn, be targeted. Repeated pecking at the skin may cause haemorrhaging. The exposed blood often attracts more birds to the area, intensifying the rate of pecking and inducing cannibalism (Savory and Mann, 1997). The damage can lead to severe injury and death. The recipient initially attempts to escape, but the persistent pecking can lead to learned helplessness, where the recipient bird appears to give up and submits to the pecking. Severe feather pecking is, therefore, often described as a behavioural disorder because of its detrimental nature (van Hierden et al., 2004; van Zeeland et al., 2009; Kops et al., 2013a). Beak trimming is widely used as a method to reduce the damage caused by feather pecking (Hartcher et al., 2015a; Hartcher et al., 2015b). However, it has severe welfare implications. The beak is highly enervated and hot-blade trimming often results in acute pain in young birds and chronic pain, and neuroma formations in older birds (Gentle et al., 1990; Gentle et al., 1991 and reviewed in Gentle, 2011). Infrared trimming has also been shown to produce acute pain, reduced activity, and less time spent eating and drinking (Marchant-Forde et al., 2008). Although beak trimming is still allowed according to European legislation, European countries such as Norway and Sweden have banned it.

Feather pecking is known to be a multifactorial behaviour, influenced by a number of environmental, and genetic factors (Hartcher et al., 2016). Discussing all of these would go beyond the scope of this thesis and genetic factors are therefore presented only superficially in section 1.3.3. Some of these factors, however, are introduced in more detail in the following sections.

1.3.1 Litter supply

Feather pecking is largely accepted as redirected ground pecking and a large number of studies have attributed the development of feather pecking predominantly to early life access to litter (Blokhuis, 1986; Newberry et al., 2007). Experimental studies have demonstrated that chicks with restricted access to litter in the first month of life show increased feather pecking in adulthood (Bestman et al., 2009). Likewise, provision of litter during the rearing period increases plumage quality and reduces feather pecking, cannibalism, and mortality of adult birds (Blokhuis and Van Der Haar, 1989; 1992; Johnsen et al., 1998; Gunnarsson et al., 1999). Furthermore, access to litter or sand during rearing can prevent the development of feather pecking in hens later transferred to

barren environments compared to rearing on a wire floor only (Vestergaard et al., 1997). Large-scale on-farm studies also report the importance of early experience with litter. Interruption and limitation of litter supply during rearing increases fearfulness, feather damage, and the incidence of severe feather pecking (Gilani et al., 2013). Hens reared on litter spend more time foraging and less time feather pecking (Huber-Eicher and Sebö, 2001). In addition, access to litter in aviary systems decreases mortality (Aerni et al., 2005). There is, however, some evidence that does not support feather pecking as a redirected ground pecking. A study has found that birds doing a lot of feather pecking also showed more ground pecking, particularly in larger group sizes (Bilcik and Keeling, 2000). One explanation is that feather peckers are more active in general. Indeed, the activity of the group and feather pecking have been positively correlated (Savory and Mann, 1997). A recent observational study has suggested that the use of litter, simply as a layer of paper, on which dust and droppings can accumulate, can have benefits in reducing feather pecking and feather damage (de Haas et al., 2014b). However, no experimental work has tested the effects of provision of paper on the development of feather pecking in the full scale, real life context, of egg farming.

1.3.2   Other environmental factors

Several studies suggest an effect of the environment on the prevalence of feather pecking. Lighting is a relevant factor, with severe feather pecking and mortality levels being greater in higher light intensity while gentle pecks are more frequent in low light intensities (Kjaer and Vestergaard, 1999). Overall, studies suggest that feather pecking is less frequent in "enriched" environments. Indeed, the provision of bundles of twine string, or "pecking devices", has been shown to hold the interest of chicks even after extended exposure and decrease the frequency of pecking behaviours (Jones and Carmichael, 1999a; Jones et al., 2002; McAdie et al., 2005). Interestingly, these pecking devices and provision of whole oats and deeper litter did not result in better plumage conditions when available only from 12 days of age (Hartcher et al., 2015a; Hartcher et al., 2015b). Supplying flocks with sand, polystyrene blocks, or long-cut straw also decreases feather pecking behaviour (Huber-Eicher and Wechler, 1997; 1998). In addition, frustration due to thwarting of an expected resource or reward can increase the pecking rate (Haskell et al., 2000). Among non-beak-trimmed birds, housing in pens with litter, nest boxes, perches, and at lower stocking density, results is less

feather pecking compared to housing in conventional cages (McAdie and Keeling, 2002). Furthermore, the availability of an outdoor run with artificial or vegetative cover reduces the incidence of feather pecking and is associated with better plumage conditions (Bestman and Wagenaar, 2003; Heerkens et al., 2015). Due to this understanding that feather pecking is less frequent in enriched environments, the environmental enrichment provided by production farmers was included as a factor that might influence the incidence of feather pecking in paper **IV**.

1.3.3 Genetic factors

Feather pecking has also been seen to occur independently of environmental factors because of a genetic predisposition. Red jungle fowl, the ancestor of domestic chickens, have also been observed to feather peck (Jensen et al., 2005). Hens from the Rhode Island Red origin are less fearful and develop less feather pecking compared to hens from a White Leghorn origin (Uitdehaag et al., 2008). Dekalb white hens have been shown to be more fearful and have more feather damage compared to ISA brown hens (de Haas et al., 2013). It is possible to artificially select for a low or high frequency of feather pecking and produce different phenotypic lines of High Feather Pecking (HFP) and Low Feather Pecking (LFP) from this process (Kjaer et al., 2001; Rodenburg et al., 2004; Rodenburg et al., 2008). LFP birds spend more time foraging and feeding and less time feather pecking compared to birds from the HFP line (van Hierden et al., 2002; Rodenburg and Koene, 2003). The specific loci associated with feather pecking have been found and quantitative trait loci studies have indicated that feather pecking may be controlled by different genes in the juvenile and the adult phases (Buitenhuis et al., 2003a; Buitenhuis et al., 2003b). Feather peckers show more activity in a novel object test, open field test, and restraint test, suggesting that this trait is genetically associated with a proactive coping strategy (Jensen et al., 2005).

It has been suggested that HFP and LFP lines represent proactive and reactive coping styles, respectively (Korte et al., 1997). Proactive animals have a low adrenocortical response and an active behavioural response to a stressor, whereas reactive animals have a high adrenocortical response and an inactive behavioural response to stressors (Koolhaas et al., 1999). Accordingly, HFP individuals have lower plasma corticosterone levels compared to individuals from an LFP line (van Hierden et al., 2002). It is also possible to artificially select lines for low mortality of the

flock, which, in non-beak-trimmed birds, is often a consequence of severe feather pecking and cannibalism (Rodenburg et al., 2009; Kops et al., 2013b; Kops, 2014). Birds from the low mortality line show reduced fear and lower levels of noradrenaline and 3,4-dihydroxyphenylacetic acid (DOPAC), a metabolite of dopamine, compared to birds from a control line (Nordquist et al., 2011; Kops et al., 2013b).

1.4 Cognitive function

Cognitive abilities in general and spatial learning and memory specifically are important for the fitness and survival of mobile species. In order to navigate the environment, animals must be able to perceive, store, and retrieve information. Laying hens must find feed, water, perches, and nests and therefore require good spatial memory to remember specific routes and landmarks. However, one can imagine that these cognitive abilities are more important in complex environments. Laying hens housed in cages live with few other individuals and have all the resources available to them in a confined space, where locating each one is not a difficult challenge. In an aviary system, however, the hens must navigate a much more complex physical and social environment. Feed, water, perches, and nests are distributed across an ample three-dimensional space and thousands of conspecifics cohabitate.

The underlying mechanisms of cognitive abilities evolve in response to selective pressures imposed by environmental differences between populations and by the species' ecology (Pravosudov and Roth, 2013). This process is also relevant for domestic species under artificial selection, such as laying hens, in line with the resource allocation theory (see Schutz et al., 2002). This theory suggests that each individual has a limited amount of resources to sustain all biological activities and that individuals will optimise the allocation of their resources in order to maximise fitness. Among domesticated laying hens in a complex environment, those individuals that invest energy towards developing greater cognitive abilities will be better equipped to, among other things, navigate their specific environment. They will have better access to resources and will be able to avoid risky social interactions. These resources, in turn, enable the hens to maximise their egg output. However, developing and maintaining the neural pathways required for these cognitive functions is very costly (Isler and Van Schaik, 2009; Niemela et al., 2013). Therefore, if the environment is so simple that only less advanced cognitive abilities are needed, artificial selection

will favour animals that allocate resources preferentially to egg production. Consequently, despite the fact that laying hens are not directly selected for cognitive ability, cognitive development may be influenced by the environment to which these animals are exposed (Sol et al., 2005). As a result, according to the Predictive Adaptive Response hypothesis (see section 1.4.1), natural and artificial selection are likely to favour individuals that program the allocation of resources to cognitive function, depending on the environment encountered during the early stages of development (Bateson et al., 2014). Alternatively, the Silver Spoon hypothesis (see section 1.4.1) suggests that, in a poor environment, these advanced cognitive abilities do not develop as well simply because of the lack of proper stimulation (Grafen, 1988; Monaghan, 2008). Consequently, according to both the Predictive Adaptive Response and the Silver Spoon hypotheses, one can expect that rearing laying hens in a barren cage environment will repress cognitive development compared to rearing in a complex aviary environment. However, no experimental work has yet been done to investigate this.

1.4.1   *Predictive Adaptive Response Hypothesis and Silver Spoon Hypothesis*

It is well known that factors other than genetics influence behavioural development. Cues received early in life can dictate how the organism will respond phenotypically in order to be best adapted to the environment later in life (Bateson et al., 2014). The capability of an organism to respond to these environmental cues without changing their genome is called adaptive developmental plasticity (Bateson et al., 2014). Natural selection likely favours individuals that programme the allocation of resources depending on the environment encountered during the early stages of development. The environmental circumstances experienced early in life provide an estimate of the conditions one will most likely have to face as an adult (Monaghan, 2008; Bateson et al., 2014). The benefits of having this plasticity are explained by the Predictive Adaptive Response hypothesis (Bateson et al., 2014) (Figure 1 A). A Predictive Adaptive Response allows the organisms to adapt to the future environment as best as possible, increasing their likelihood of survival until reproduction. Likewise, a mismatch between the forecasted and actual environment can result in reduced fitness (Bateson et al., 2014). If the early and adult environments differ largely, adaptations that were vital in the early environment might become disadvantageous in the adult environment. An example of this principle comes from extensive research on the effects of postnatal maternal care in rats (*Rattus norvegicus*) on the behavioural development of the pups

(reviewed in Claessens et al., 2011). Adult offspring from mothers that performed high rates of licking and grooming show high cognitive performance and decreased behavioural and endocrine responsiveness to stress (Liu et al., 1997; Caldji et al., 1998; Liu et al., 2000). As suggested by the Predictive Adaptive Response hypothesis, adult offspring of low licking and grooming mothers (considered a form of adversity) performed better in a high-stress context compared to offspring from high maternal care mothers (Champagne et al., 2008; Bagot et al., 2009). Likewise, high maternal care offspring had better performance in a low-stress context (Champagne et al., 2008; Bagot et al., 2009). In general, fitness is highest when an adult organism lives in the type of environment in which they developed (Monaghan, 2008).

An alternative hypothesis describing the effects of early environment on developmental plasticity and adult fitness is the "Silver Spoon" hypothesis (Grafen, 1988) (Figure 1 B). This hypothesis suggests that favourable environmental conditions early in life have lasting effects causing higher fitness in individuals that experienced such environments when young compared to those that were born in poor environmental conditions irrespective of the circumstances during adulthood (Grafen, 1988; Monaghan, 2008; Douhard et al., 2014). Many examples are available from bird species. In Ural owls (*Strix uralensis*), females that are born during periods of increased food availability have better reproductive success compared to females born during periods of decreasing food availability (Brommer et al., 1998). In this case, having the advantage of favourable circumstances as young owls resulted in access to better resources and higher fitness as adults. According to the Silver Spoon hypothesis, individuals born in poor conditions consistently have worse fitness than those born in favourable conditions because of trade-offs during development (Monaghan, 2008).
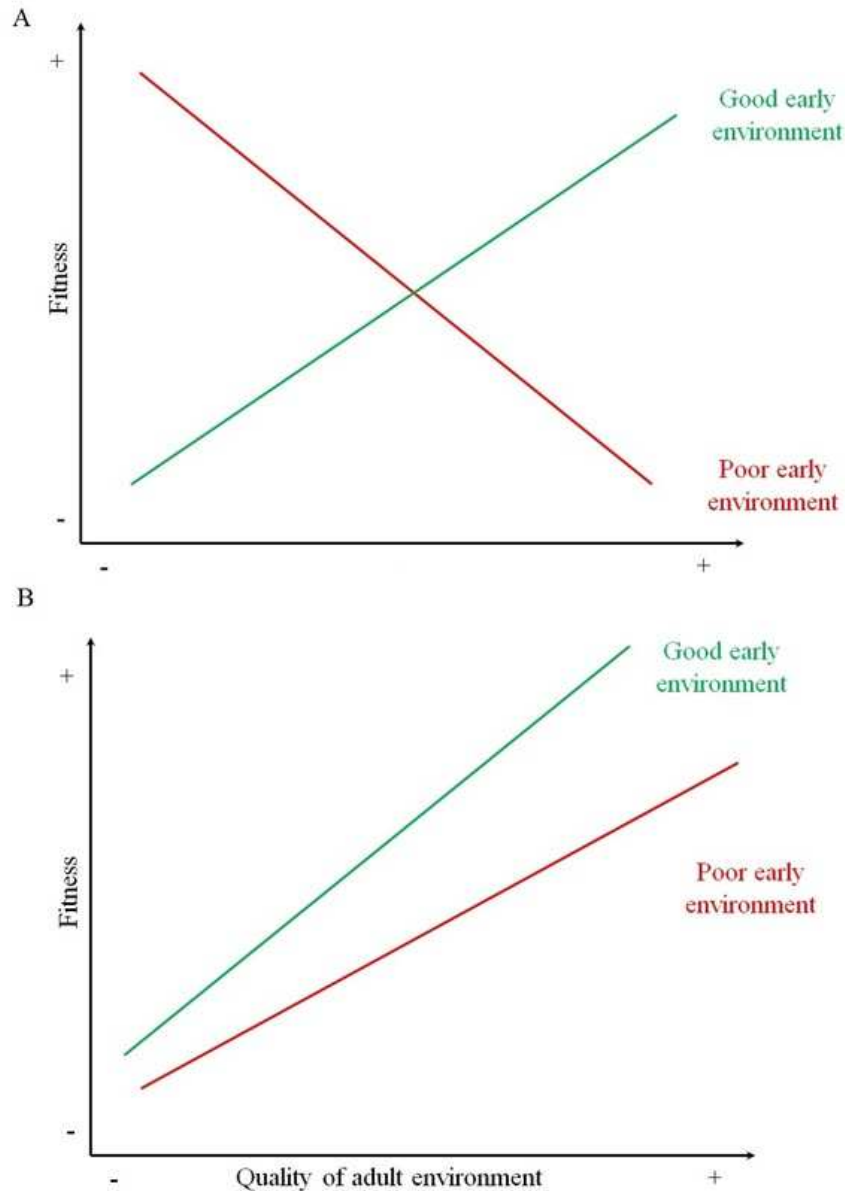
Figure 1: Figure describing the relationship between fitness (vertical axis) and the quality of the adult behaviour (horizontal axis) relative to the quality of the environment experienced early in life. (A) Predictive Adaptive Response hypothesis. In this situation, there is environmental matching. The fitness of an individual will be highest when the quality of the adult environment is similar to the environmental quality experienced early in life, due to adaptation to those circumstances. **(B) Silver Spoon hypothesis.** The fitness of all organisms increase with higher environmental quality; however, those organisms that developed in a good quality

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environment will always have higher fitness compared to those that were born in poorer conditions. Adapted from Monaghan (2008).

For laying hens in Norway, the environments experienced during rearing and during adulthood can be vastly different. However, these environments do not vary in quality along a "poor-good" scale, such as poor and good food availability or high and low predation levels. Instead, they vary in complexity and, consequently, the physiological and behavioural qualities the hens must have to make full use of the resources in each environment. These different levels of environmental complexity subject the birds to different pressures and obstacles they must manage in order to optimise their biological function.

According to the Predictive Adaptive Response and Silver Spoon hypotheses, fitness is affected by the conditions of the early life environment and the (mis)matching between young and adult environments (Monaghan, 2008; Bateson et al., 2014). In a similar manner, laying hen welfare may also be affected by the environmental conditions in the young and adult periods of life. For example, a hen reared in a simple cage environment may not need highly developed spatial memory to find and access resources and may, therefore, allocate energy to other biological functions rather than the development and maintenance of specific neurological pathways (see section 1.4). This adaptation is perfectly suitable for a simple environment. However, if the hen is later transferred to a more complex environment, it might not have the spatial cognitive ability necessary to navigate the terrain and find and access resources. Indeed, a study has shown that hens reared for the first two months of life without access to perches have impaired spatial skills as adults and were not able to access food rewards in certain locations (Gunnarsson et al., 2000). In this example, the relationship between young and adult environmental complexity and welfare behaves much like what would be predicted by the Predictive Adaptive Response Hypothesis for the relationship between environmental quality and fitness. Likewise, hens reared in complex aviary environments and later transferred to a furnished cage laying system have to cope with the new restrictive environment and may have reduced welfare due to negative emotions such as frustration according to the framework of the Predictive Adaptive Response hypothesis.

These examples illustrate that it might be possible to use the theoretical framework of the Predictive Adaptive Response and Silver Spoon hypotheses to compare the relationship between young and adult environmental complexity and welfare, even though the hypotheses were created on the premise of environmental quality and fitness (see section 5.2).

1.4.2 Proximate mechanisms for the effect of the environment on cognitive functions

Evidence of positive effects of enriched environments on solving cognitive tasks is available from previous studies in birds (Pravosudov et al., 2006), rodents (Lyst et al., 2012), and fish (Spence et al., 2011). Studies of domestic chickens have also shown that differences in the early rearing environment have pronounced and long-lasting effects on spatial skills (Gunnarsson et al., 1999; Gunnarsson et al., 2000). In addition, the literature shows that more complex environments and experiences in the juvenile phase cause a growth of the brain, particularly of the hippocampus, the brain area involved in spatial cognition and memory. A study of marsh tits (*Parus palustris*) indicates that the brain of birds that had the experience of food caching had a larger hippocampal region and more hippocampal neurons compared to the brain of birds that did not experience caching and food retrieving (Clayton and Krebs, 1994). Another study showed that the hippocampus of migratory birds increases in size when the birds have experienced migration, compared to individuals that do not experience migration (Pravosudov et al., 2006).

The effect of environmental experience on cognition may also be via changes in the dopaminergic pathways, which are known to affect working memory in several species including species of birds (Herold et al., 2008), nonhuman primates (Brozoski et al., 1979; Sawaguchi et al., 1990) and rodents (Zahrt et al., 1997). Dopamine D1 receptors in the prefrontal cortex are fundamental for the expression of brain-derived neurotrophic factor (BDNF), which is involved in synaptic plasticity essential for memory formation (Nieoullon and Coquerel, 2003; Suzuki et al., 2012; Xing et al., 2012). Dopamine D1 receptor knockout mice have been shown to have spatial learning deficits (El-Ghundi et al., 1999; Xing et al., 2012). Knockout of D3 receptors, however, improves spatial memory (Nakajima et al., 2013) and emotional memory (Micale et al., 2010). Furthermore, a study in adult laying hens has demonstrated that more environmentally complex housing conditions increase the hippocampal cell soma size and result in a left-skewed asymmetry in the density of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine

(Nagatsu et al., 1964; Patzke et al., 2009). It is not known, however, whether differences in housing conditions during the rearing period affects TH in the chicken brain. Box A describes the process of synthesis and metabolism of dopamine in the brain.

BOX A. Synthesis and Metabolism of Dopamine

Dopamine is synthesised from the non-essential amino acid tyrosine (Figure 2; reviewed in Fernstrom and Fernstrom, 2007; Daubner et al., 2011). The enzyme Tyrosine Hydroxylase catalyses the hydroxylation of tyrosine to dihydroxyphenylalanine (L-DOPA) (Nagatsu et al., 1964). L-DOPA is then decarboxylated to dopamine by the enzyme Aromatic L-Amino Acid Decarboxylase (Holtz, 1939 in Nagatsu et al., 1964). When a dopaminergic neuron is depolarised during an action potential, dopamine is released from presynaptic vesicles as neurotransmitters into the synaptic cleft and binds to dopamine receptors on the postsynaptic cell membrane (reviewed in Beaulieu and Gainetdinov, 2011). From the synaptic cleft, dopamine is often recycled by dopamine transporters back into the presynaptic neuron (reviewed in Wightman and Zimmerman, 1990). The catabolism of dopamine results in Homovanillic acid (HVA) (reviewed in Eisenhofer et al., 2004; Figure 2). Dopamine can be metabolised to 3, 4-dihydroxyphenylacetic acid (DOPAC) by the enzymes Monoamine Oxidase A and B (MOA-A/MOA-B) (Richter, 1937; Erwin and Deitrich, 1966 in Eisenhofer et al., 2004). Alternatively, dopamine is metabolised to 3-methoxytyramine (3-MT) by Catechol-O-methyl-transferase (COMT) (Eisenhofer et al. 1995). Finally, these metabolites are further metabolised to HVA, which is excreted by the kidneys and liver (Anggard et al., 1974; Eisenhofer et al., 1995).
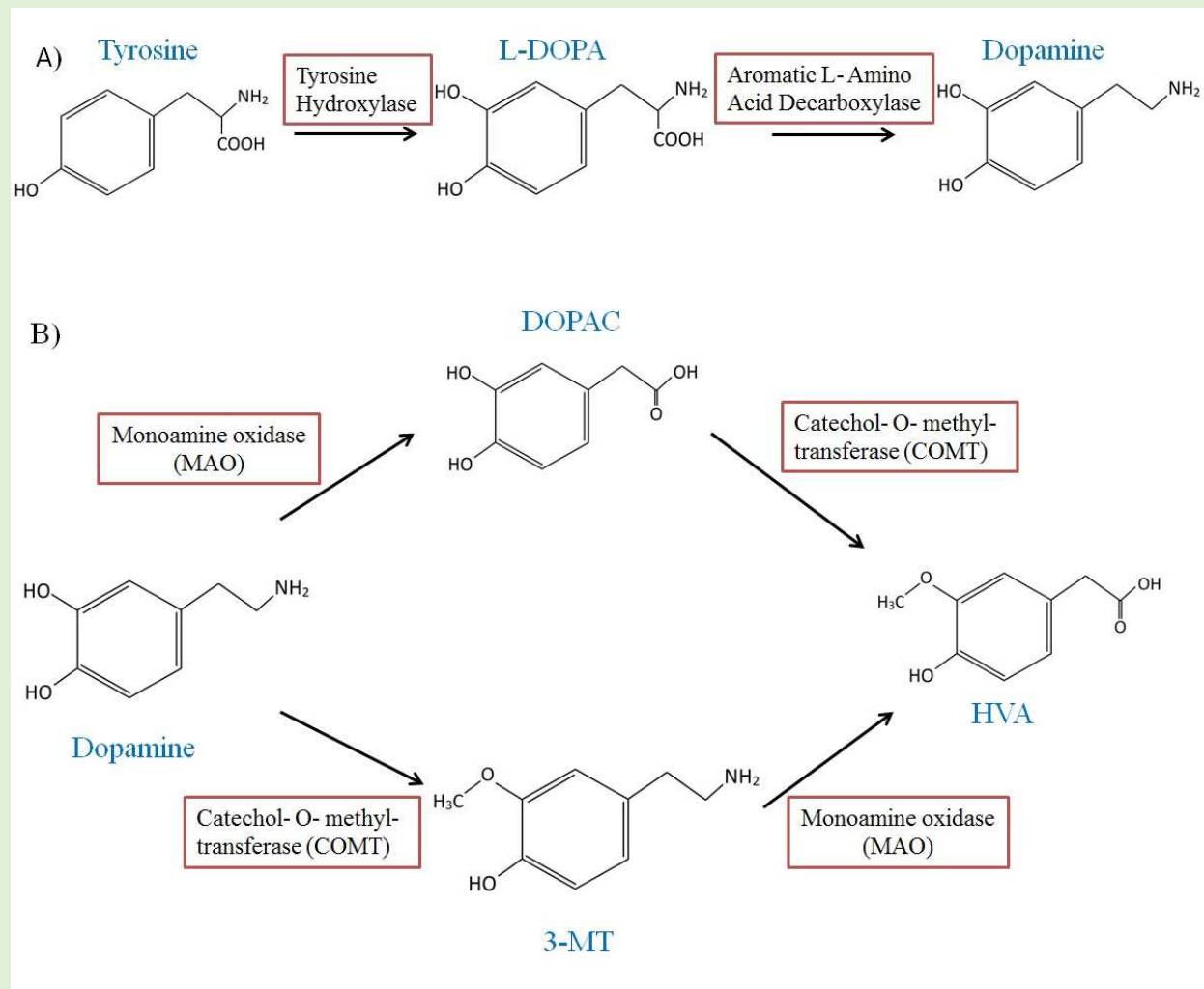
Figure 2: Synthesis (panel A) and metabolism (panel B) of Dopamine. (Modified from (Eisenhofer et al., 2004; Daubner et al., 2011).

1.5 Knowledge gaps

The domestic chicken is a widely used animal species for food production. As mentioned, close to 7 billion laying hens are produced worldwide per year in the farming industry (FAO, 2013). Therefore, it is particularly important to investigate the welfare implications of farming husbandry procedures.

It is not known how the welfare and productivity of these animals respond to disparities between the rearing and adult environments. Nevertheless, the practice of housing adult aviary-reared hens in furnished cages is somewhat common. This knowledge gap was addressed in this thesis. Furthermore, despite all that is known about feather pecking and the factors that influence it, particularly in regards to litter supply, no experimental work had ever been performed to test whether provision of paper substrate in the aviary rows from the first day of age reduces the incidence of feather pecking in adult laying hens in an aviary farm setting. This was another of the knowledge gaps this thesis aimed to fill.

Finally, also as a major model organism for both basic and applied avian research (Rose, 2000), it is not known how the two contrasting environments of aviary- and cage-rearing systems influence the development of cognitive functions in the laying hen. The work performed in this thesis aimed at investigating this topic, from the behavioural and proximate/mechanistic points of view.

2. Aims and Objectives

The aim of this thesis is to increase knowledge of the effects of the environment during early life on the behavioural development and welfare of laying hens during the production period. In order to achieve this aim, this thesis has the following objectives.

- Objective 1: Welfare and Productivity

  The aim of this sub-goal was to establish whether birds reared in aviaries and producing in furnished cages show behavioural indicators of poorer welfare than did birds producing in furnished cages after rearing in traditional rearing cages (Paper **I**).

- Objective 2: Spatial Memory

  This part of the project aimed to test whether rearing in aviary or cage systems influences spatial cognition in laying hens (Paper **II**).

- Objective 3: Neuroanatomy

  The aim of this sub-goal was to test and describe the long-term impact of early environmental complexity on tyrosine hydroxylase in the chicken hippocampus and the caudolateral nidopallium, the avian functional analogue to the mammalian prefrontal cortex (Paper **III**).

- Objective 4: Feather Pecking

  The aim of this study was to describe the effects of access to a paper substrate from early rearing on the incidence of feather pecking and feather damage during the production stage (Paper **IV**).

3. Materials and Methods

3.1 Housing conditions

3.1.1 Experimental animals

In the studies presented in this thesis, two breeds of domestic chicken (*Gallus gallus domesticus*) were used as experimental animals. All animals were non-beak-trimmed and of normal health. In papers **I** and **IV**, female Lohman-selected leghorn chickens of ages 0-30 weeks (in paper **IV**) and 0-21 weeks (in paper **I**) were studied within a commercial setting. In papers **II** and **III**, female white Dekalb chickens of ages 0-23 weeks (in paper **II**) and 0-24 weeks (in paper **III**) were studied in an experimental setup.

3.1.2 Rearing conditions

For all papers, rearing took place at commercial farms and, aside from any specific treatments, all other husbandry procedures such as feeding, lighting and temperature, were standard and followed recommendations from the management guide of the relevant layer breed. In paper **I**, the birds were incubated and hatched at the same time in the same hatchery. They were then reared in one of two rearing treatments: in an aviary- or in a conventional cage-rearing system. Birds in the two treatments were provided with the same feed but were housed in different rooms containing either aviaries or rearing cages at the same farm. At 16 weeks of age, 7500 hens, half from each rearing treatment, were transported to the same furnished cage production farm.

In papers **II** and **III**, the birds were hatched at a commercial hatchery and reared in separate corridors in a single room until 16 weeks of age (Figure 3). Each corridor had either a cage- or an aviary-rearing system. This system consisted of cages stacked in three tiers on either side of a corridor, allowing inspection by the caretaker. All cages could be opened at the front allowing the birds to move between tiers and the floor of the corridor. To increase the pullets' ease of access, ramps ran from the floor to the second tier. When the doors were opened, perches extended from the front of the first and second tiers. On arrival at the rearing farm at one day of age, all birds were placed within the aviary rows with the doors closed. At four weeks of age, the doors of half

of the house were opened, allowing the animals to move freely within their corridor and tiers. This was the aviary-rearing group. The other half of the cages remained with the doors shut and the birds enclosed inside, resulting in a cage-rearing system. The animals remained in their respective environments until 16 weeks of age.

In paper **IV**, 12 rearing farmers were assessed for eligibility. Of these, five had the appropriate facilities for the study design and agreed to be enroled into the study. Among the five farms included, approximately 489,000 laying hens were randomly allocated to one of the two treatment groups. The rearing farmers were asked to close the divisions between the aviary rows of the system in order to stop the animals from moving between corridors, effectively forming two separate groups within the same house. In one of these groups, the rearing farmers supplied the cages in the rows with chick paper over the floor netting from the time of arrival to the rearing farm until the age when they are let out onto the floor of the system (i.e. at 5 to 6 weeks of age). The chick paper makes it easier for the young animals to walk on the wire mesh without getting their legs caught. It also allowed the collection of particles such as dust, spilt food and droppings, thus providing the chicks with foraging substrate inside the aviary row from the first day of age. For the control group, situated in another row within the same house, no paper was supplied. Thus, the animals in the control rows were standing on bare netting inside the cages until the day they were let out onto the floor. At five to six weeks of age, the side doors to the aviary rows were opened for both treatment groups and the animals were allowed to move freely within their corridor. At 16 weeks of age, the hens were transported from the rearing farms to production farms, where they were visited by the researchers for data collection. Only aviary production farms were visited for the purpose of this study. Each production farm received hens from only one rearer and from only one treatment group. Any production farms that received mixed flocks were excluded from the study.

Figure 3: Photograph of one unit of the rearing system used in papers II and III. The left side of the image shows housing conditions for aviary-reared birds and the right side shows the conditions for cage-reared birds. Photo credit: Andreas Salte.

3.1.3 Experimental housing

In paper **I**, birds from both rearing treatments were transported from the rearing farm to the same production farm at 16 weeks of age. The housing at the production farm was furnished cages (designed for housing 10 hens according to EU requirements) and contained eight to nine birds per cage according to Norwegian legislation. 7,500 birds, half of which came from each rearing treatment, were included in the study. The groups were kept separate so that the composition of each cage was not mixed but either contained birds rearing in conventional rearing cages or birds reared in aviary systems. The cages were tiered within the house creating three levels of cages, arranged in four rows. Each row contained either aviary- or cage-reared birds. For papers **I** and **IV**, all other husbandry procedures were standard and followed recommendations from the Lohmann Management Guide (Lohmann, 2014).

In paper **II**, 24 birds from each treatment (N = 48) were transported from the rearing farm to the experimental facilities at the Norwegian University of Life Sciences campus Adamstuen, Oslo. There they were group housed in Victorsson T10 furnished cages. Each cage contained two aviary-reared and two cage-reared birds. In paper **III**, at 16 weeks of age 40 birds from each treatment (N = 80) were transported to the poultry facilities at the Norwegian University of Life Sciences, campus Ås, Norway. Here they were housed in custom-built pens. Each pen contained one nest box, an elevated platform at 110 cm height and two perches, one at 70 cm height and one at 140 cm height. Birds were housed in mixed groups of six aviary-reared birds and six cage-reared birds per pen. For papers **II** and **III**, all husbandry procedures were standard and followed the recommendations from the Dekalb Management Guide (ISA, 2009).

In paper **IV**, each treatment and control flock from each rearing farm was transported to an aviary production farm. No mixed flocks were included in the study. The hens were treated as any other production flock. Most often production farmers were not aware that the hens were participants in a research project. Those farmers that knew were blinded to which treatment the flock belonged. The flocks were visited by the researchers at around 30 weeks of age, the peak of lay. During the visits, the hens were assessed for pecking behaviour and for feather damage.

3.2 Feather damage and feather pecking

In total, 23 aviary production farms were visited for paper **IV**. Each farm contained on average 7500 laying hens from the same rearer and from the same treatment group (paper or control). The same two researchers visited each production farm once when the hens were around 30 weeks of age, the peak of lay. This time point was chosen as an age where birds are settled into the production environment, the laying percentage is at its highest and feather quality should be optimal (no feather damage due to advanced age). Both producers and researchers were blinded to which experimental treatment the visited flock belonged. During this visit, which lasted two to three hours, the hens were assessed for signs of feather pecking via inspection of the plumage and by direct observation of pecking behaviour. In addition, during the visitation to production farms, the researchers made notes on the use of environmental enrichment.

3.2.1 Feather damage

In paper **IV**, feather damage was assessed individually on sight in 40 to 100 hens per flock. This assessment method was adopted from the Welfare Quality® Assessment protocol for laying hens (Welfare Quality, 2009). It was decided that the assessment should be done on sight, rather than by handling the birds, to minimise stress and disturbance. Choice of hen was based on the following principle: one hen was chosen and the second closest to that hen was visually scored. Only hens that had all assessed body parts visible to the observer were scored. The observer walked calmly along the corridors and scored hens from all parts of the house (floor, slats, ramps, perches, etc.). Scores were awarded using a three-point score in three different body parts: head/neck, back/rump and belly/cloaca. Each area was given a score from A to C. A score "A" was awarded to areas with no or only slight wear of the feathers. "B" was given when the area had at least one patch of naked skin less than 5 cm wide. Areas with at least one patch of naked skin wider than 5 cm were given the score of "C". These individual areas' scores were then added to yield a total feather score for each bird as follows: if all three areas were scored "A" the total feather score was 0 (zero). If the hen had received at least one "B", but no "C"s, the total score was 1. At least one "C" would result in a total feather score of 2. The individual scores were used in the analysis.

3.2.2 Feather pecking

In paper **IV**, direct feather pecking observations were carried out in two different locations within the house, one close to the outer wall of the house and one in the centre corridor. In each location, the observer sat down quietly and waited until most of the hens in the area ($\geq 80\%$) no longer directed their attention towards the observer, i.e. did not point either eye at the observer. Following this, the observer selected an area of approximately 1 m^2 on the floor and noted any events of gentle and severe feather pecking within that area during 20 minutes. Gentle feather pecking was defined as nibbling and gentle feather pecks without reaction from the receiver. Severe feather pecking was classified as forceful pecks with attempts to pull feathers from the recipient's body, generally leading to a withdrawal response of the receiver. The observer noted how many of each type of pecking were observed.

3.3 Behavioural observations

3.3.1 Home cage observations

Home cage observations were performed in paper **I**. The flock at the production farm was visited on two separate occasions during the laying period, once at 19 weeks and again at 21 weeks. During both visits, a total of 99 cages from both rearing treatments were recorded with the use of hand-held cameras mounted on tripods. No cage was filmed more than once. After recording started, the researcher left the house for ten minutes before returning to place a novel object, an empty water bottle, attached to the inside front bars of the filmed cages. The researcher then left the room containing the birds and recording continued for a further ten minutes. Subsequently, the researchers returned to remove the novel objects and the cameras and assembled them in a different location within the house. The video recordings were later viewed with Observer XT 7.0 software for behavioural analysis of indicators of comfort and aversion and of alert behaviour towards the novel object.

3.3.1.1 Behavioural indicators of comfort and aversion

In paper **I**, the behavioural analysis was conducted by a single researcher who was blind to the rearing background of the birds. Observations commenced after one minute of recording to avoid recording behaviour of the birds in the presence of the researcher. The birds in the cage were numbered and a focal animal was selected at random. In the event of the focal animal moving out of sight, the chicken immediately to its right became the focal subject and was observed subsequently. The behaviours noted are presented in Table 1. For preening, bout length was measured as well as frequency and total duration. For the remaining variables, only the frequency was recorded.

Table 1. Ethogram of comfort and aversion-related behaviour

Behaviour	Description
Flap wings	Bilateral wing movement including wing raising
Stretch wings	Unilateral backward and downward stretching of leg and wing together
Dust bath	Lie on side, scratch at cage floor, rub head and neck on floor, open wings.

Feather raise	Raise feathers with or without rigorous rotation of body around axial plane, subsidence of feathers back to smooth position
Preen	Raise feathers and clean or realign them with beak
Scratch self	Leg brought upwards and forwards under wing to scratch lowered head
Tail wag	Rapid sideways movement of tail
Shake head	Rapid rotary movement of head, accompanied by slight raisin of head and neck feathers

3.3.1.2 Alert behaviour towards a novel object

In paper **I**, the animals' alert behaviour was measured in relation to the duration of time the focal bird remained in the zone closest or farthest from the novel object and whether the bird directed its attention to the novel object (Table 2). Observations for alert behaviour started one minute after the placement of the novel object into the home cage. The focal animal was selected in the same method as for observations of comfort and aversion behaviours. In the event of the focal animal moving out of sight, the bird directly in front of it was selected as the new focal animal so to avoid influencing the duration of occupation in any given zone. All variables were recorded continuously and were mutually exclusive.

Table 2. Ethogram of alert behaviours definitions of proximity to novel object

Behaviour	**Description**
Near to novel object	Subject's head occupies the half of the cage housing the novel object
Far from novel object	Subject's head occupies the half of the cage farthest from the novel object
Alert behaviour	Neck extended vertically, either eye oriented toward novel object. Includes alert behaviour in both sitting and standing positions, but sitting as a component of nesting or dustbathing not included. Extended neck behaviours for drinking are not included

3.3.2 Holeboard maze

The holeboard maze (paper **II**) can be used to measure working memory, general working memory, and reference memory in a number of situations following a habituation period. It is an open field arena containing nine identical small cups equidistant to each other in a 3×3 matrix (Figure 4). The cups sat on top of thin wood-coloured plates. Prior to habituation, the birds were taught that food rewards in the form of mealworms could be found inside the small cups. During the habituation period of five days, all the cups in the maze contained one mealworm each and the birds were given the opportunity to explore the maze in pairs, for the first day, and alone, for the last four days, for five minutes. All 48 birds were habituated to the maze. Their performance in speed and efficiency at eating all the mealworms was recorded and the 24 best birds (one aviary-reared and one cage-reared from each cage) were selected for use in the testing phase.

During the training and testing period, only three out of the nine cups contained mealworms. In the first, uncued acquisition phase of the test, each bird entered the maze alone in twice daily trials for 14 days. The configuration of baited cups each bird experienced was always the same during this phase. During this phase, the birds were trained to find the three mealworms in the room without the aid of any cues to distinguish baited from unbaited cups. The trials lasted a maximum of five minutes and were terminated early in the event of the bird eating all three mealworms within that time. In the second phase, the configuration of baited cups remained the same as before and cues were added to the baited cups, so they could be easily distinguished from the others. This was achieved by placing red wooden plates underneath the baited cups. During this phase, which lasted five days, the birds also visited the maze alone in twice-daily trials of maximum five minutes but these were terminated early when the three mealworms were found. In the third phase, the over-training phase, the cues were removed from under the baited cups and the maze returned to the same state as during the uncued acquisition phase. This phase also lasted five days. The fourth and final phase, the reversal phase, lasted four days. Here the configuration of baited cups changed and there were no cues to distinguish baited from unbaited cups. This introduced a change that required the birds to replace the previous information regarding the configuration of the rewarded cups with information about the new configuration.

The following measures were noted and/or calculated for each trial: trial duration, working memory, general working memory and reference memory. Trail duration was defined as the total duration of the trial until all mealworms had been eaten or the maximum of five minutes has elapsed. Working memory was defined as the ratio of rewarded visits to the number of visits to the baited holes. General working memory was defined as the ratio of the number of unique visits to the total number of hole visits. Reference memory was defined as the ratio of the number of visits to the baited holes to the number of visits to all holes. For each individual, the average of each of the four measures was calculated per phase and this average score used for statistical analysis.

Figure 4: Photograph of holeboard area. Panel A: holeboard setup configuration as used during uncued acquisition, over-training and reversal phases. Panel B: representative configuration of the holeboard setup for the cued acquisition phase. The red wooden plates mark baited cups. Photo credit: Fernanda M. Tahamtani.

3.4 Physiological methods

3.4.1 Blood sampling

Blood sampling was performed for measurement of blood glucose concentrations in paper **I**. The sampling occurred on the last day of the two visits to the production farm after all the behavioural data had been collected. The blood samples were collected from 24 different animals per treatment per visit. Only one hen per cage was used for this procedure.

The hen was taken out of the cage by one researcher and held while the comb was pricked with a Haemolance lancet to produce a drop of blood. The drop was collected on the strip of an Accu-

Check Mobile glucose monitor and values were read directly from the monitor. The duration of the procedure from the collection of the bird to the removal of blood was ≤ 1 min.

3.4.2 Brain dissections and immunohistochemistry

Anti-tyrosine hydroxylase immunohistochemistry of the chicken hippocampus and caudolateral nidopallium (NCL) was the subject of paper **III**. The brains were dissected at 20 and 24 weeks of age after the birds had been sedated and euthanised by cervical dislocation. After immersion fixation with paraformaldehyde and 30% sucrose, the brains were frozen and stored at -80°C. Later the brains were cryosectioned in the frontal plane and laid on Superfrost slides. The hippocampus and NCL were identified with the use of a brain atlas for 2-week-old chickens (Puelles et al., 2007) taking into account the increased brain size for hens at 20 and 24 weeks of age. Immunohistochemical detection of TH was performed using 5% normal goat serum for blocking and incubated with rabbit polyclonal tyrosine hydroxylase antibody. Negative controls were incubated with rabbit immunoglobulin fraction. The staining was visualised using 3'3-diaminobenzidine (DAB).

3.4.3 Tissue imaging

In paper **III**, the immunohistochemical staining of TH in chicken brains was imaged with the ZEISS software ZEN Pro 2012, Blue edition, (ZEISS, Germany), which was used for imaging with a Zeiss Imager M2 AX10 microscope and Zeiss Axiocam 506 colour camera. Hippocampal measurements were undertaken in two distinct areas, the medial hippocampus (mHp) and the dorsomedial hippocampus (dmHp), whereas the NCL was analysed as a whole. Regions of interest (ROIs) were selected under ×10 magnification using the contours function (Figure 5), and the average pixel intensity in each ROI was calculated. The background pixel intensity per section was also measured from an area of no staining in the section. The values from the ROIs were then corrected for variability in staining per section by subtracting these values from the background intensity. As intensity is on an inverted scale (high-intensity levels mean low levels of staining), this background correction results in the difference between background intensity and staining intensity, a measure that is directly proportional to staining level.
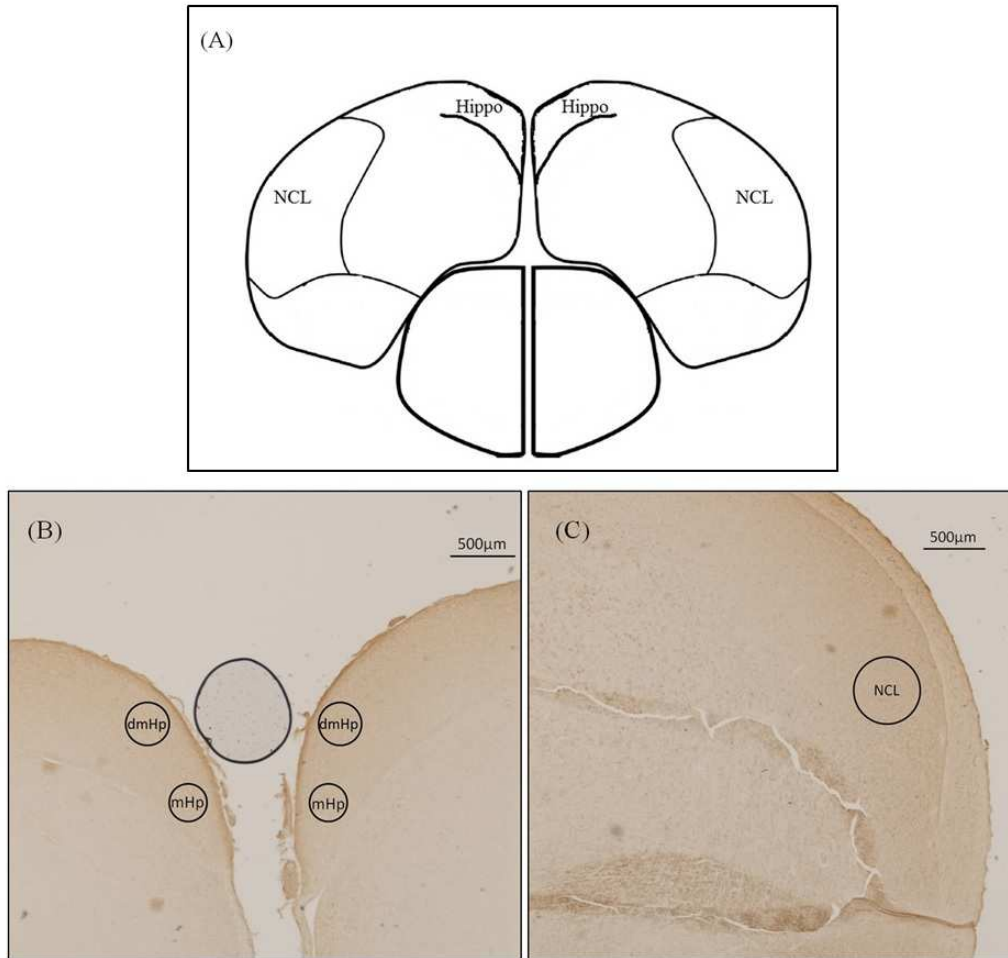
Figure 5: Representative tyrosine hydroxylase immunohistochemical staining with the three analysed areas. Panel A: Schematic drawing of the chicken brain sectioned along the coronal plane illustrating the hippocampus (Hippo) and caudolateral nidopallium (NCL) (interaural 2.56 mm). Panel B: Photograph of the dorsomedial hippocampus (dmHp), medial hippocampus (mHp). Panel C: Photograph of caudolateral nidopallium (NCL). Photo credit: Fernanda M. Tahamtani.

3.5   Production data

In paper **I**, production data were collected by the producer and were summarised for 20, 24, 28, 41 and 73 weeks of age. These data included egg production, average egg weight and egg quality illustrated by the number of eggs with hairline cracks. Hen mortality was noted throughout the production period until euthanasia at 76 weeks of age.

3.6 Statistical analysis

In paper **I**, comfort behaviour was comprised of long bouts of preening (over 2 seconds long), wing flapping, wing stretching, dustbathing, feather raising and tail wagging. Aversion-related behaviour was comprised of short bouts of preening (up to 2 seconds long), self-scratching and head shaking. Due to a large number of birds not performing comfort or aversion-related behaviours, the data did not conform to the assumptions of the general linear model (GLM) and therefore an ordinal variable was created to indicate whether a bird showed comfort or aversion-related behaviour or not. This variable was used for analysis instead. The effects of treatment on the number of birds showing comfort and/or aversion-related behaviours was then analysed using ordinal logistic regression in a model including the effects of rearing treatment, cage height (bottom, middle or top) and the interaction between treatment and cage height.

Long and short bouts of preening were analysed separately. An ordinal variable was also created for this data set, because of a large number of zeros, indicating whether the birds performed long and/or short preening or not. The modelling procedure was the same as explained above. The duration of alert behaviour performed in the half of the cage closest to the novel object conformed to the assumptions of the GLM. Therefore, ANOVA was used to test the effects of rearing and cage height, as well as the interaction between these two factors, on the duration of alert behaviour. Data for blood glucose concentration were normally distributed and analysed with the Student's t-test to compare rearing treatments. The results for hen mortality, egg production, and egg quality data are reported as chi-squared and p-values.

In paper **II**, a repeated measures ANOVA was used to test the effect of rearing environment on the four parameters (trial duration, working memory, general working memory and reference memory), with bird as random factor nested in treatment, and treatment and phase as fixed factors, as well as the interaction between treatment and phase. In paper **III**, the effect of rearing environment on the TH staining intensity was tested using repeated measures ANOVA, with brain ID as a random factor nested in treatment and in pen, and treatment, age and hemisphere as fixed factors. The interactions between treatment and age, age and hemisphere, treatment and hemisphere and treatment age and hemisphere were also included in the model. The fixed factor

room was also tested but found to be insignificant for each dependent variable and thus removed from the final model.

A short description of all response and explanatory variables investigated in paper **IV** are presented in Table 3. In paper **IV**, the counts of gentle and severe feather pecking were analysed using Negative Binomial Regression. The final models for both gentle and severe feather pecking included treatment (control vs. paper), enrichment and rearing farm as fixed factors. The feather score data were analysed using a mixed multilevel ordered logistic regression model, using production farm and rearing farm as random effects to account for lack of independence between data points within farms. Feather score was the dependent variable with score 0, 1 and 2 as the three alternatives, and the final model included the fixed factor treatment (control vs. paper) and farmer and rearer as random effects with farmer nested in rearer. The results are reported as the odds ratio of having a higher feather score for the control compared to the paper treatment group. All statistical analyses were performed using JMP® versions 9.0 to 11.1.1 (SAS Institute Inc., Cary, NC, USA) and Stata SE 14 (StataCorp LP).

Table 3. Description of all response and explanatory variables investigated in paper **IV** (Savory 1995)

| Response variables	Description
Gentle feather pecking	Nibbling and gentle feather pecks without reaction from receiver Pecks/min
Severe feather pecking	Forceful pecks with attempts to pull feathers from the recipient's body, generally leading to a withdrawal response of the receiver Pecks/min
Feather damage score	Overall damage score from zero (no damage) to 2 (bad damage)
Explanatory variables	
Treatment	Experimental treatment as applied during the rearing period
Rearing farm	Farm responsible for the rearing period. A total of six rearing farms participated in this study
Enrichment	Presence or absence of environmental enrichment at the production farm
System	Classified as "open" or "closed". Refers to the availability of floor space and ease of movement of hens within the production house
Production farm	Farm for the production period. A total of 23 farms were included in this study

4. Synopsis of results

4.1 Paper I

This study aimed to establish whether birds reared in aviaries and producing in furnished cages showed behavioural indicators of poorer welfare than birds producing in furnished cages after rearing in traditional rearing cages. It was hypothesised that, due to the effect of frustration and stress caused by environmental restriction, birds reared in aviaries would show poorer welfare than those raised in rearing cages. Hens from both rearing treatments were transported, at 16 weeks of age, to the same furnished cage production house. They were visited twice during the production period and observed for comfort behaviours, aversion-related behaviours and alert behaviour towards a novel object. Data were also collected from each group on production parameters and blood glucose concentration. Aviary-reared birds performed more alert behaviour towards a novel object than cage-reared birds at 19 but not at 21 weeks of age (Figure 6). In addition, aviary-reared birds in the cages in the lower tier tended to perform more comfort behaviour than cage-reared birds. There was no difference in aversion-related behaviour performance or in blood glucose levels between the treatment groups. However, aviary-reared birds had higher mortality than cage-reared birds with 209 dead aviary birds compared with 94 dead cage birds throughout the production period.
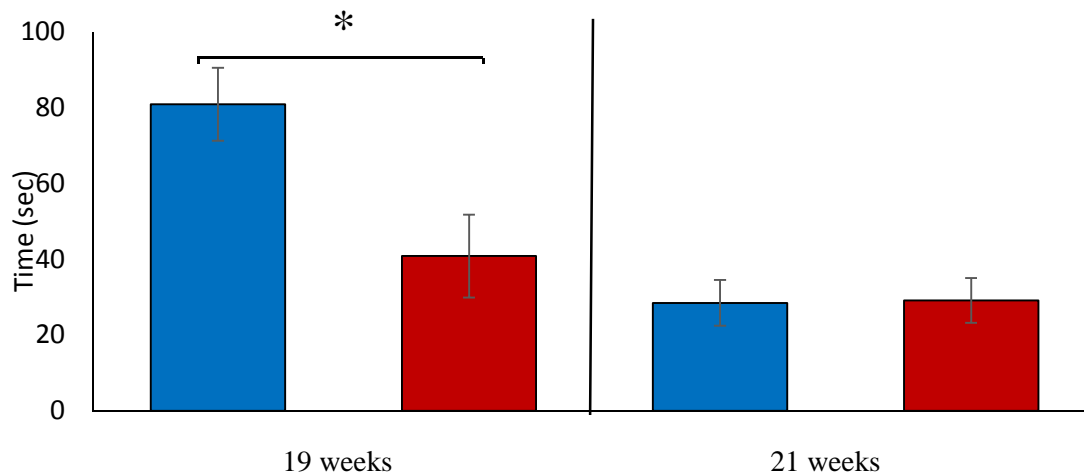
Figure 6: Results from paper I. Mean time (seconds) spent showing alert behaviour in aviary- (blue bars) and cage- (red bars) reared birds at 19 and 21 weeks of age. Significant differences marked *.

4.2   Paper II

In paper **II**, the effects of aviary and cage rearing on spatial cognitive functions were described. The aim was to test the hypothesis that rearing in a barren cage environment relative to a complex aviary environment causes long-lasting deficits in the ability to perform spatial tasks. Laying hens were reared in two groups in the same room but with different levels of environmental complexity. 24 birds, 12 from the aviary treatment, 12 from the cage treatment, were then trained and tested in a holeboard task. All but three chickens, two from the cage-reared treatment and one from the aviary-reared treatment, searched for bait in the holeboard. The birds that did not search the holeboard were excluded from analysis. Mean trial duration for both treatment groups decreased during training. Likewise, as a whole, the memory component performance (working memory, general working memory and reference memory) increased with training but dropped when tested in the reversal phase, for both treatments. Overall, there were no treatment effects on trial duration or any of the memory components. There were, however, interaction effects of treatment and phase. During the reversal phase, when the learned bait configuration was changed, the cage-reared chickens took longer to complete the holeboard task than aviary-reared chickens. Furthermore, aviary-reared birds had better working memory performance during the reversal phase compared to cage-reared birds.

4.3   Paper III

In paper **II**, results from the holeboard maze demonstrated a behavioural effect of differential levels of rearing environment complexity in cognitive ability, specifically in memory. Therefore, the study presented in paper **III** was devised to further investigate this at the neurochemical level and attempt to understand the proximate causation of these results. Brains (N=67) were dissected from birds that received the same rearing treatments as the birds used in paper **II** at 20 and 24 weeks of age. The brains were then prepared for immunohistochemical detection of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine. The areas analysed were the medial hippocampus (mHp), dorsomedial hippocampus (dmHp) and caudolateral nidopallium (NCL). In the mHp, the right hemisphere of aviary- and cage-reared birds of both ages had higher staining intensity for TH compared to the left hemisphere. There was also a tendency for brains at 24 weeks of age to have more TH compared to younger brains at 20 weeks of age. No

effects were found on the TH staining intensity in the dmHp. In the NCL, the results also indicated an effect of the hemisphere on TH staining intensity, this time with the left hemisphere having higher levels compared to the right. There was also an interaction effect between treatment and age. However, this effect was lost after *post hoc* testing.

4.4   Paper IV

The primary aim of paper **IV** was to test the efficacy of provision of a paper substrate during the rearing period on the reduction of feather pecking in a commercial farm setting. Neither gentle nor severe feather pecking was found to be affected by treatment (control or paper). However, both were found to be affected by rearing farm. In addition, production farms that provided environmental enrichment had flocks performing less gentle feather pecking at 30 weeks of age compared to production farms that did not provide enrichment, regardless of which treatment the flocks received during rearing. The results of the feather score show that the odds ratio for having a higher feather score category for the control compared to the paper treatment group was 3.28. This means that the estimated odds of having a higher feather score, i.e., poorer feathers, is 3.28 times as large for a bird from the control treatment compared to a bird from the paper treatment. Overall, birds from the paper treatment group had a higher probability of having feather score 0 and a lower probability of having feather score 1 or 2 than the control birds. Thus, the effect of paper could be seen on all levels of feather damage score.

5. Discussion

5.1 Environmental complexity and laying hen behaviour and physiology

In paper **I**, it was seen that in the early weeks after transfer to the furnished cage production farm, aviary-reared birds performed more alert behaviour towards a novel object than cage-reared birds. As previously mentioned, alert behaviour towards a novel object is associated with positive choice and is, therefore, an indicator of positive welfare (Nicol et al., 2011b). The finding that aviary-reared birds performed more alert behaviour than cage-reared birds was, therefore, surprising as it was expected that aviary-reared birds would be stressed and/or frustrated by the restrictive environment and would show behavioural signs of poorer welfare. Instead, these results suggest that these hens have better welfare than cage-reared hens in the early weeks after transfer to production in furnished cages. The aviary-rearing environment exposes the birds to a greater number of novel situations and possibilities for escaping unwanted situations. For example, in an aviary house, the farmer often has to remove some of the litter that accumulates on the floor of the aviary every few weeks. This activity may involve bringing into the aviary wheelbarrows, shovels, buckets and even new personnel. In a cage system, this type of activity is not necessary, as all litter is collected on and removed by the litter belts positioned under the cages. The aviary environment also presents a larger space to explore and more conspecifics with which to interact. As seen in paper **II**, the density in a cage-rearing system (25 birds/m^2) is higher than that in an aviary-rearing system (12 birds/m^2). Therefore, the greater space available to the hens allows them to escape situations they would rather avoid (Sandilands et al., 2009). Chronic stressors that cannot be predicted or avoided generally result in depression-like symptoms referred to as learned helplessness, which is normally characterised by a lack of responsiveness to external stimuli (Taylor et al., 2001; Richter et al., 2013; Vollmayr and Gass, 2013). Freedom of movement in aviary-reared hens is likely to provide them with an experience of having control over their surroundings, which would reduce the risk of developing learned helplessness. Indeed, it has been shown that access to perches in loose housing systems reduces aggression and cloacal cannibalism because lower-ranking/pecked hens use the perches to escape higher-ranking/pecker conspecifics (Sandilands et al., 2009). However, learned helplessness was not assessed in the current study so it is not possible to affirm that learned helplessness was a factor behind the treatment effects

observed. Nevertheless, as a sign of positive choice (Nicol et al., 2011a; Nicol et al., 2011b), the alert behaviours observed in paper **I** indicate that aviary-reared birds demonstrate a better capability to cope with environmental change than cage-reared birds, and experience better welfare, at least during the first three weeks after transfer from rearing to production environment. The combination of intermittent exposure to novelty and the experience of having control over their surrounds is likely to cause long-term changes in personality that result in a better ability to cope with environmental change (Carere et al., 2005).

The frequency of performance of comfort behaviours overall, across both rearing treatment groups, was low. Wing stretch was only observed four times while wing flapping and dustbathing were not observed at all. This was expected, as it is known that the frequency of comfort behaviours in furnished cages is low, even at low stocking densities, when each individual has more space in which to perform such behaviours (Albentosa and Cooper, 2004). Nevertheless, the results showed that, at 19 weeks but not at 21 weeks, aviary-reared birds perform more comfort behaviour in the bottom tier compared to cage-reared birds. Considering the instinctive anti-predator behaviour of perching and roosting high from the ground (Newberry et al., 2001), this result suggests that aviary-reared birds experience better welfare than cage-reared birds when prevented from moving away from the ground.

These results on comfort and alert behaviours suggest that aviary-reared birds have better welfare than cage-reared birds, but only during the first few weeks after transfer from rearing to a furnished cage production house (i.e. at 19 weeks of age). At 21 weeks, no such differences were found. Furthermore, the time aviary-reared hens spent showing alert behaviour is numerically lower at 21 than 19 weeks (Figure 6). This is important as it indicates that the two groups are more similar at 21 weeks of age because the welfare of aviary-reared birds decreased compared to at 19 weeks while the welfare of cage-reared birds remained stable. This notion is further corroborated by the results of this study showing that mortality was higher in aviary-reared hens (5.52%) compared to cage-reared hens (2.48%) throughout the laying period. Overall, these results are interesting because they suggest that aviary rearing produces more robust chickens with better coping mechanisms, but that these attributes are not sufficient to cope with the restrictive environment in the long term. In addition, these results indicate that hens reared in traditional rearing cages seem

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to have better survival under the conditions of a furnished cage production system, but they do so under poorer levels of welfare than would be possible. This is indicated by higher performance of alert behaviour towards a novel object by aviary-reared birds, compared to cage-reared birds, three weeks after transfer to the furnished cage production farm (paper **I**). As discussed in section 1.1.1, furnished cages were developed in an effort to improve opportunities for behavioural expression while maintaining the economic and health advantages of cage housing. Mortality due to disease or injury has impacts on both physical health and mental well-being (Cockram and Hughes, 2011). Therefore, the high mortality of aviary-reared hens in paper **I** suggests that aviary-reared hens experienced poorer welfare (both poor physical health and poor mental well-being) when transferred to a furnished cage production system compared to cage-reared hens.

Blood glucose concentration is used as an indicator of stress as it increases as a result of corticosterone secretion from the adrenal cortex following activation of the hypothalamic-pituitary-adrenocortical (HPA) axis (Simon, 1984; OnbasIlar and Aksoy, 2005). Blood glucose concentrations have also been validated as an indicator of welfare based on their negative association with positive choice (Nicol et al., 2009). In paper **I**, no difference between the blood glucose concentrations of aviary and cage reared birds was found. This was contradictory to our prediction that aviary-reared birds would be frustrated following transfer to the more spatially restrictive environment. These results do not completely support the findings from the behavioural observations, which indicate that aviary-reared birds have better welfare than cage-reared birds at the third week following transfer between systems. It is, however, likely that behaviour is a more sensitive measure of the birds' response to environmental change than activation of the HPA-axis.

In paper **II**, the results from the holeboard task indicated that aviary-reared birds have better working memory compared to cage-reared birds. Working memory is considered a form of short-term memory while reference memory is considered a form of long-term memory (Bimonte-Nelson et al., 2003; Xing et al., 2012). Working memory contains elements that are trial dependent – "what has happened, when and where" such as which holes have been visited – and helps the bird avoid revisits and maintain an effective foraging strategy (van der Staay et al., 2012). It must, therefore, be reset after each trial so as not to influence performance in the next trial (Ordy et al., 1988; Frick et al., 1995). Cage-reared birds had lower levels of working memory than aviary-

reared birds during the reversal phase. This difference in short-term working memory indicates that rearing in a barren environment adversely affects working memory. It is noteworthy that the repeated training in a cognitive task for a six-week period does not compensate for the cognitive deficit caused by early life in a relatively impoverished environment. A previous study indicates that the first two months of life without access to perches is enough to impair the cognitive skills necessary to move around a three-dimensional space in laying hens tested at 16 weeks of age (Gunnarsson et al., 2000). In addition, a recent study found that aviary-reared birds make more use of perches and elevated platforms compared to cage-reared birds (Brantsæter et al., 2016a). The reduction in working memory in cage-reared birds at the reversal phase, seen in conjunction with the corresponding elevated latency to eat all the mealworms seen in paper **II**, may indicate that these individuals are more sensitive to environmental change than aviary-reared birds. Therefore, the results from paper **II** might suggest that aviary-reared birds are more resilient to environmental change compared to cage-reared birds. It is important to note that differences in trial duration between the two treatments were only observed during the reversal phase. This indicates that the cage-reared birds had difficulty in finding the food rewards due to the change in the configuration of baited cups, but not due to any limited mobility resulting from rearing in the space-restricted environment of a rearing cage.

Similar to the results from paper **II** comparing aviary- and cage-reared laying hens, domesticated laying hens show a reduction in spatial learning performance compared to the wild ancestor, red jungle fowl, due to artificial selection for high productivity (Lindqvist and Jensen, 2009). Domestication and selection for high productivity reduce the need to spend energy searching for food, as it is made freely available, and results in the allocation of more resources to production traits (see Andersson et al., 2001; Lindqvist and Jensen, 2009). In paper **II**, we see that the complexity of the aviary environment requires some allocation of energy to cognitive functions as feed and other resources are more difficult to access compared to the more simplistic cage environment.

Unfortunately, the results from paper **III** did not shed light on the proximate mechanisms of the environmental complexity effects on working memory. We did not find any treatment effects of aviary- and cage-rearing on the staining intensity of TH in the chicken hippocampus and NCL. The chickens were reared in an aviary or in cages until 16 weeks of age, at which point they were

transported to experimental facilities and were housed in equal pens of intermediate complexity between aviary and cage systems. They remained in this type of housing until brain dissections at 20 and 24 weeks of age. It is possible that during the period of experimental housing, any differences between the rearing groups disappeared. It is also probable that an environment more complex than an aviary, such as a free-range system, could produce the expected differences in TH in laying hens (Patzke et al., 2009). An alternative reason for the lack of the rearing effects on TH is that dopamine is not the sole modulator of memory formation. Future research in this area should look for changes in other elements of cognitive mechanisms of memory formation such as cell soma size, brain-derived neurotrophic factor, NMDA (N-methyl-D-aspartate) receptors, and components important for long-term potentiation (see section 5.5).

The results from papers **I** and **II** taken together suggest that aviary rearing produces hens that are more robust and resilient to environmental change and more capable of coping with common stresses that result from husbandry procedures such as transfer to a production environment. Indeed, in a parallel study to paper **I**, we also found that aviary-reared hens showed lower levels of fearfulness compared to cage-reared hens in the first five weeks after transfer to furnished cages (Brantsæter et al., 2016b). In addition, a recent study showed that aviary-reared birds are less fearful towards a novel object and a human observer compared to cage-reared birds (Brantsæter et al., 2016a). However, the results from this thesis also suggest that aviary-reared hens may not be capable of coping with the restrictive environment of furnished cages in the long term and that cage-reared hens may find difficulty in navigating a complex aviary environment due to cognitive deficits.

5.1.1 *The role of husbandry procedures on the development of feather pecking*

In paper **IV**, depriving laying hens of access to foraging substrate until after the first five weeks of age resulted in poorer plumage quality as adults which is indicative of feather pecking. The paper introduced to the aviary row cages at one day of age allows the accumulation of feed, waste, and dust particles, creating a litter area and providing opportunities for expression of aspects of the appetitive phase of feeding behaviour, such as foraging and food manipulation. As discussed previously, hens are highly motivated to peck and forage. Domestication and selection for high

productivity resulted in layer breeds ingesting food at higher frequencies than the wild ancestor (Andersson et al., 2001). Studies with blind and sighted hens indicate that domestic hens are both internally and externally motivated to peck (Sandøe et al., 2014). Therefore, chicks learn to peck even when no appropriate pecking material is available. Furthermore, the process of learning to peck and manipulate objects can be rewarding even if not related to consumption (Hogan, 1973). All these factors reinforce exploratory pecking behaviours and, therefore, withholding the possibility of doing so can lead to frustration or suffering caused by deprivation (Dawkins, 1988). Frustration is a negative emotion that animals will avoid if they can and affects welfare negatively (Dawkins, 1990). In laying hens, thwarting of feeding behaviour by providing visible but unobtainable food causes a fear response, which results in stereotyped pacing and short bouts of preening, and increases the concentration of faecal corticosterone metabolites (Duncan and Wood-Gush, 1972a; Duncan and Wood-Gush, 1972b; Janczak et al., 2007). These behavioural consequences of frustration can be reduced with the administration of anti-anxiety medication (Duncan and Wood-Gush, 1974). Furthermore, hens exhibit an increase in frustration-induced pecking towards conspecifics due to thwarting access to a reward or expected resource (Haskell et al., 2000), and recent studies suggest that experience with litter that later becomes unavailable increases the frequency of feather pecking (Gilani et al., 2013; de Haas et al., 2014b).

As a negative emotion, frustration impairs the "feel good" aspect of welfare as defined by Webster et al. (2004). The Brambell report (1965) also considered frustration as an important aspect of animal welfare:

"The degree to which the behavioural urges of the animals are frustrated under the particular conditions of the confinement, must be a major consideration in determining its acceptability or otherwise".

The results from paper **I**, showing that aviary-reared birds suffered higher mortality in a furnished cage production farm compared to cage-reared birds, may also indicate that these birds were suffering from frustration. Anecdotally, a large proportion of the birds that were found dead had bloody sores on the head and neck region indicative of injurious pecking. This suggests that the aviary-reared birds may have been more susceptible to the development of injurious pecking than cage-reared birds due to frustration caused by lack of appropriate foraging substrate. Similarly, the

birds from the control treatment in paper **IV** developed worse feather conditions as adults, likely due to feather pecking that became habitual, possibly as a result of thwarted motivation to forage during the first five weeks of life. Therefore, the results from papers **I** and **IV** suggest that certain husbandry procedures may induce frustration in the animals, causing deficits in both welfare and production parameters. Hens reared in aviaries spend the first 16 weeks of life in a complex environment with free access to foraging substrate. Housing aviary-reared hens in a furnished cage environment is comparatively restrictive and without ample opportunity to express foraging motivations. Similarly, chicks reared without access to litter for the first five weeks of life seem to develop feather pecking as a redirected foraging behaviour due to large innate motivation to forage. In both cases, the consequences are observed weeks after initial, and continuing, frustration (paper **I**) or after said frustration has been terminated (paper **IV**). This large interval between initial frustration and when the behaviours were observed suggest that the experience of frustration, of being thwarted from performing motivated behaviours, influences the development of the hens, for example developing feather pecking behaviour, and that these changes may be long-term. In light of the results from paper **IV** showing more feather damage in hens that had not been given access to paper from one day of age (control group), compared to hens from the treatment group, it is surprising that no corresponding increase in feather pecking behaviour was observed in the control group. This may be a consequence of the sensitivity of the recording method used. Feather pecking behaviour in this study was measured at only one relatively short time period, at 30 weeks of age for 20 minutes. This method is relatively short term and only gives information from that one period in time. Feather damage, on the other hand, happens over a longer period.

It is important to note that the higher mortality of aviary-reared birds in paper **I** may have also been due to a decrease in physical health. A disadvantage of aviary environments is that they ease the transmission and spread of diseases and parasites, compared to cage systems (Tauson, 2002; 2005). Therefore, it is possible that the aviary-reared hens had higher mortality due to disease. Unfortunately, it was not possible to specify the cause of death in this study as a post-mortem assessment was not performed.

It is worth noting that in paper **IV**, both gentle and severe feather pecking were observed, during direct observation, in both treatment groups to some degree. Perhaps this is indicative of the poor

quality of the litter available. Farmers rarely supply the aviary corridors with foraging material such as wood shavings, straw and hay, or chopped cardboard. Instead, they often rely on the accumulation of dust, feathers, and faeces produced by the animals themselves. Some production farmers provide environmental enrichment, such as empty ice cream boxes, hanging CDs from the system, and Siporex blocks. As seen in paper **IV**, providing such material reduced the incidence of gentle feather pecking. This is in accordance with the literature, which suggests that current substrate is vital for the inhibition of feather pecking. A study showed that hens housed on wood shavings perform less feather pecking and more ground pecking than hens housed on a wire floor, irrespective of previous experience with litter (Nicol et al., 2001). Furthermore, other studies have tested the effects of modified management, such as not lighting the inside of nest boxes, having a radio playing inside the hen house, and including roosters in the flock, and found that these modifications reduce feather pecking and feather damage (Zimmerman et al., 2006; de Haas et al., 2014a). These results suggest that adult laying hen behaviour is somewhat flexible and greatly influenced by the current environment (Nicol et al., 2001; Zimmerman et al., 2006; de Haas et al., 2014a).

The results from paper **IV** also suggest that overall rearing procedures may affect the incidence of feather pecking as indicated by the significant effect of rearing farm on both gentle and severe feather pecking. This indicates that the rearers' specific management, routines, and attitudes as a whole can have an impact on the development of feather pecking. If laying hens are so motivated to forage, and suffer frustration when deprived of foraging, as the literature suggests, the need to provide a substrate of constant quantity and quality should be clear.

5.2 Consequences for welfare (PAR or Silver Spoon?)

In section 1.4.1, the concepts of the Predictive Adaptive Response hypothesis and the Silver Spoon hypothesis were introduced, each having a slightly different view on how early environment, adult environment, and fitness interact (Grafen, 1988; Bateson et al., 2014). As mentioned, much like fitness, welfare may be affected by any behavioural and physiological consequences of young and adult environmental (mis)match. The development of behavioural disorders, such as feather pecking, negative emotions, such as fear and frustration, and the inability to find and access

resources have clear welfare implications, both for physical health and for mental well-being. It is possible, therefore, without having had the aim of testing these hypotheses, to interpret the results from this thesis in their theoretical framework to investigate how the experienced and current environment influence animal welfare. It was found that interpreting the results of this thesis in the framework of these two hypotheses might help draw conclusions as to what is the best way to rear and house laying hens for their welfare.

The results of this thesis work in large corresponds with what is suggested by the Silver Spoon hypothesis. Adult birds reared in more complex environments (paper treatment in paper **IV**, aviary rearing in papers **I** and **II**) had indicators of better welfare than birds reared in barren environments. Birds reared in complex environments had better plumage (paper **IV**), performed more comfort behaviours and alert behaviours towards a novel object for a few weeks after transfer to production farm (paper **I**), and demonstrated better working memory performance in a cognitive test (paper **II**). These results from papers **I** and **II** suggest that rearing in a complex aviary produces more resilient and robust hens that are better equipped to cope with changes in the environment (see section 5.1). Other studies found that a complex aviary rearing reduces fearfulness in laying hens compared to rearing in a barren cage environment (Brantsæter et al., 2016a; Brantsæter et al., 2016b). On the other hand, the result that aviary-reared hens had higher mortality, and therefore poorer welfare, in furnished cages compared to cage-reared hens (paper **I**) points to the environmental mismatching suggested by the framework of the Predictive Adaptive Response hypothesis. It supports the notion that changes that are adaptive under certain conditions often become maladaptive under different conditions (Monaghan, 2008). The aviary-reared hens in paper **I** were better able to cope with the transfer to furnished cages, but not to live there for an extended period. Alternatively, the cage-reared hens lived longer in the furnished cages, but did so under worse mental well-being, particularly during the first few weeks after transfer. It seems, therefore, that elements of both hypotheses are seen in these results, perhaps with the Silver-Spoon-like framework having the most support in a short-term period (e.g. weeks after transfer) and the Predictive-Adaptive-Response-like pattern in the long-term (e.g. several months in a different environment).

With the results presented in this thesis it is possible to make a graph, based on the ones reported in Figure 1, but with welfare and adult environmental complexity as the axes and complex and barren rearing as the curves (Figure 7). It is difficult, however, to imagine the barren rearing curve with precision. In paper **IV**, we saw that improving environmental complexity (i.e. providing enrichment) was important in decreasing the incidence of feather pecking, regardless of which rearing treatment the birds experienced. Indeed, this is also supported by previous literature that suggests that current litter supply is more important to the inhibition of feather pecking than whether litter was supplied in the past or not (Nicol et al., 2001). Furthermore, modified management during housing such as providing pecking blocks, having a radio playing in the hen house, and adding roosters to the flock reduces feather damage (de Haas et al., 2014a). This points to the barren rearing curve being slightly higher than the complex rearing curve in adult environments of lower complexity and transitioning to slightly below the complex rearing curve, but still increasing in welfare, as the adult environment becomes more complex (Figure 7 A). On the other hand, perhaps it would be impossible for an individual with barren rearing to cope with a complex adult environment. The results from paper **II** show that barren rearing causes long-term impairments in cognitive skills. Hens reared in cages might not have the mental capability to appropriately find and make use of resources if they are sent to an aviary system during the production period. Indeed, a previous study showed that laying hens reared for the first 8 weeks of life without access to perches had higher difficulty in reaching food rewards at elevated heights compared to hens reared with access to perches (Gunnarsson et al., 2000). In addition, this impairment of the hens' spatial skills was seen at 16 weeks of age (Gunnarsson et al., 2000), eight weeks after hens from both groups were given free access to perches. This further suggests that deficits in spatial cognition are long lasting and can prevent the animals from accessing important resources for several weeks and months. This would cause the barren rearing curve in a welfare vs. environmental complexity plot to decrease much more sharply as adult environmental complexity increases (Figure 7 B).
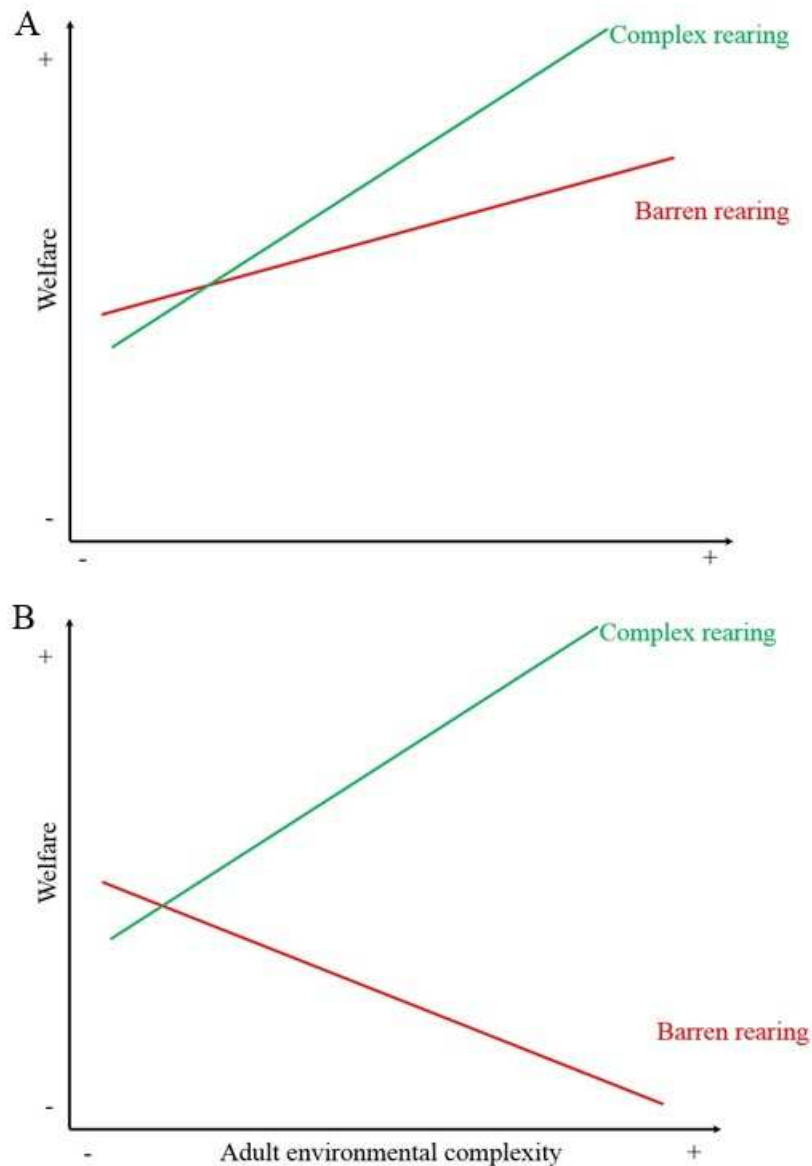
Figure 7. Two alternative relationships between environmental conditions during rearing and during adulthood and their effects on welfare. Welfare is presented on the vertical axis, adult environmental complexity on the horizontal axis. The complex rearing curve and the barren rearing curve are presented in green and red, respectively. Panel A: Birds reared in complex environments have slightly poorer welfare in barren adult environments compared to birds reared in barren environments (higher performance of comfort and alert behaviours but

higher mortality in aviary-reared birds in paper **I**). Meanwhile, birds reared in barren environments have increased welfare as the complexity of the adult environment increases (reduction in gentle feather pecking in the presence of enrichment, paper **IV**). **Panel B:** The complex rearing curve behaves the same as in panel A, but the barren reared birds experience a decline in welfare with the increase of complexity, due to inability to cope/find resources (barren rearing causes deficits in working memory, paper **II**).

It might have been possible to draw the barren rearing curve with more accuracy had we performed a crossover study in which both aviary and cage-reared hens had been transferred to, and housed in, both an aviary and furnished cage production systems. This study design would have made it possible to compare how birds reared in a barren environment cope with the transfer and adaptation to an environment vastly different and more complex than what they had been used to. However, it would have been difficult to find an aviary production farmer willing to do this.

Of course, to have a truly complete image of how laying hen welfare is affected by the rearing and adult environment combination, it would be necessary to look not only into blood glucose concentrations, and behavioural and production indicators, as done in this thesis work, but also at other physiological and health indicators as well. For example, aviary-rearing facilitates coccidiosis (*Eimeria* spp) vaccination through successive natural exposure to antigens (Farr, 1943). On the other hand, because in a cage environment the wire mesh allows infected faeces to fall out of reach of the birds, coccidiosis vaccination is not effective in cage-housed birds (reviewed in Reid, 1990; Price, 2012). Therefore, cage-rearing may be a health risk factor for the development of coccidiosis, particularly if the hens are later housed in an environment where they have contact with pathogens (Broussard et al., 1986; Frame and Bickford, 1986). Another example is bone weakness and fractures. Aviary systems promote exercise and greater freedom of movement, which increases bone strength (McLean et al., 1986), but also promotes more bone fractures (Kappeli et al., 2011 and reviewed in Sandilands et al., 2009). Perhaps a combination of aviary rearing a furnished cages production might produce stronger bones and fewer breaks.

In any case, the results from this thesis suggest that sending hens to produce in an environment different from that which they experienced during rearing has implications for animal welfare and

even production parameters. The findings from paper **I** indicate that aviary-reared chickens are less suitable for transfer and production in a cage system as illustrated by their higher mortality in the furnished cage production system compared to hens that were reared in cages. Norwegian legislation requires that all hens be reared with access to perches and substrate material (Landbruks- og matdepartmentet, 2001). A small proportion of rearing farmers has kept their conventional cage rearing systems but installed perches and dustbathing areas to fit the legal requirements. However, most rearing farmers currently use aviaries systems (Steinsland, pers. comm.). Therefore, most Norwegian hens are reared in aviaries and later transferred either to production aviaries or to furnished cages. Transfer from the rearing system to the production system is an unavoidable consequence of housing systems for laying hens that are currently used. This transfer, just as the introduction of a species to any new environment, entails new challenges to the birds, including modification of behaviour to suit the new social and physical surroundings, discovery and use of new resources, and even avoidance of previously unknown enemies (Sol et al., 2005). Therefore, it is important that more is known about the nature of laying hen cognition to improve the welfare and production of these animals.

5.3   Methodological considerations

When performing on-farm studies, researchers often rely on the participation of farmers in the administration of appropriate treatments and data collection. This arrangement, however, lacks control and supervision and often introduces more opportunity for errors. In paper **IV**, all rearing farmers were visited prior to the start of the study and instructed on how to proceed and administer the rearing treatments for the next two batches of chicks they would rear. Due to practical and economic reasons, it was impossible for all rearing farms to be visited a second time during the progression of the study. However, the researchers were available via phone/email to answer any questions the rearing farmers might have had. Still, several reared flocks had to be excluded from the study due to errors such as sending reared flocks to production farms with furnished cages, rearing chickens of another breed, and unreliable bookkeeping. Accordingly, data from the production farms could only be included if the farmer allowed the researchers to have access to the animals and perform the data collection protocols, which was not always the case. In paper **I**, the collection of production and mortality data was performed by the production farmer, during

the entirety of the production period. However, collecting of production data per treatment (aviary- or cage-reared) was only performed a few times, and not regularly, as would have been ideal. In addition, data were not always collected in a manner that allowed statistical analysis to be carried out. Egg weight, for example, was not measured individually per egg, but as a mean of 720 eggs per treatment. Therefore, it was impossible to run corresponding statistical analysis on these data. In addition, anecdotal notes on any markings found on dead birds during the production period were also taken, but not regularly. Furthermore, as the farmer only inspects the cages once a day, the bodies of dead birds were not removed from the cages until several hours after death. Therefore, it was not possible to discern with certainty whether the markings observed (often signs of injurious pecking) were administered pre- or post-mortem. Furthermore, no post-mortem assessment was performed, making it impossible to say what was the cause of death.

In paper **IV**, we used the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009). However, the protocol in its entirety takes six to seven hours to complete, which would not have been feasible. Therefore, the protocol was used only in the assessment of plumage condition. The protocol often calls for hens to be picked up and manually assessed. This procedure was considered, despite the fact that the protocol does not require manual inspection for the assessment of feather damage. In the present study, however, the decision was made to assess the hens visually only, without any handling, to avoid major disturbance and stress of the flock (and of the farmer). The limitation of this method is that plumage assessment had to be made at a certain distance, making a clear distinction between scores A, B or C for each body area harder and perhaps less accurate. However, as a parallel study of the effects of rearing treatments on fearfulness was being conducted at the same time, it was decided that not disturbing the flock was of utmost importance.

In paper **II**, the chickens were habituated to the housing facilities and to the holeboard task, including the room, the cups, and the handling by the researcher, prior to training and testing. However, they were not habituated to the cues (i.e. the red wooden plates placed under the baited cups during cued acquisition). This was an oversight as the wooden plates were always in the holeboard arena, under all the cups, but they are turned with the red side facing down. Therefore, the hens were habituated to the wooden plates but not to the red colour. This likely caused the first, and perhaps second, trial of the cued acquisition to be affected by fear and hesitation of the animals

to approach the baited cups. Nevertheless, it was clear that the birds soon learned the cues posed no threat and performed the task accordingly.

In paper **III**, the neuroanatomical effects of early environmental complexity were investigated following the results from paper **II**, showing that rearing in barren cages causes a long-term deficit in working memory. However, treatment effects in the staining intensity of TH in the hippocampus or the NCL were not found. A possible explanation for the lack of rearing effects stems from the different inclusion criteria of the two studies. In paper **II**, hens included in the study were selected after a week of habituation training and any further hens that did not learn the task were excluded from analysis. In the paper **III** study, such procedures were not possible. It is likely, therefore, that the collection of brains studied in paper **III** represents a wider range of intrinsic cognitive abilities. Furthermore, during the design of the thesis project, power calculations for sample size were performed. Alpha was set to 0.05, the standard deviation to 1, the difference to detect was set to 1.5 standard deviations and the minimum acceptable power required set as 80 percent. With these numbers, it was determined that the sample size per group would be a minimum of nine birds. As the sample size per group in paper **III** was 20, the power calculated by JMP® version 11.1.1 (SAS Institute Inc., Cary, NC, USA) was 99 percent. Therefore, lack of power was unlikely to be the reason for not detecting treatment effects.

Originally, it was planned that measurements of cell soma size and counts of cells undergoing apoptosis would be noted for the brains areas of interest, in addition to TH staining intensity. I went as far as performing the appropriate number of sections from the dissected brains and staining these sections with Cresyl Violet. However, it was clear from the start of the cell soma measurements that this data collection would take too long. Therefore, unfortunately, this line of investigation was dropped and only the immunohistochemistry staining of TH was reported.

Another issue worth mentioning is the use of a polyclonal antibodies for the detection of TH. Polyclonal and monoclonal antibodies are produced differently and have different characteristics (reviewed in Lipman et al., 2005). Monoclonal antibodies are produced by identical immune cells, all cloned from a unique parent cell. As a result, they are monospecific antibodies. Polyclonal antibodies are produced by several different immune cells and, therefore, recognise a host of

antigenic epitopes. These antibodies are more robust against slight changes in the structure of the epitope, such as genetic polymorphism and denaturation and, therefore, have higher affinity and better detection rates. However, this makes polyclonal antibodies not ideal for quantification studies, as more than one antibody may bind to the same molecule of the antigen, via different epitopes. The choice to use a polyclonal antibody in paper **III** was a practical one. Monoclonal antibodies have an inherent problem of working well in some assays and being poor or ineffective in others, a phenomenon called assay restriction. The polyclonal antibody used in paper **III** had been previously tested in our lab, among other antibodies for TH, and was the one that yielded the best results. The reason why this antibody works is likely to do with the fact that it is polyclonal. As antibodies are rarely produced for epitopes found in chickens, often the target epitopes are from humans or rodent models, monoclonals frequently do not work well with chicken samples. For non-rodent and non-human species, polyclonal antibodies often provide the best results.

5.4   Conclusions

In order to fill some of the gaps in our knowledge of laying hen welfare, the work presented in this thesis aimed to increase insight into how the rearing environment affects behavioural development and welfare. The results from paper **IV** support the current literature on the development of feather pecking due to redirected ground pecking (Blokhuis, 1986; Newberry et al., 2007; Bestman et al., 2009). Providing a paper substrate in the aviary rows from the first day of age decreases the likelihood of feather damage in adult laying hens compared to rearing without access to paper substrate. Furthermore, provision of environmental enrichment during the production phase reduced the incidence of gentle feather pecking, regardless of rearing treatment. Therefore, these simple husbandry procedures have the potential to improve laying hen welfare by reducing feather pecking.

In part, paper **I** aimed to give indications of which type of rearing system, aviary or cages, is the most adequate for ensuring the welfare of hens destined to produce in furnished cages. Aviary-reared hens had better welfare at three weeks post transfer to furnished cages, compared to cage-reared hens, as indicated by the results of alert behaviour towards a novel object and comfort behaviours in the lowest tier of cages. These results suggest an increased ability to cope with

environmental change. However, the higher mortality of aviary-reared birds in the long term suggests that their later welfare, both mental well-being and physical health, may be compromised. Furthermore, no treatment effects on blood glucose concentration, an indicator of HPA-axis activation, were found. These findings preclude the possibility of drawing general conclusions regarding which rearing method is most suitable for ensuring the welfare of laying hens in furnished cages.

The results from paper **II** suggest that rearing in a barren cage environment relative to a complex aviary environment causes a long-lasting deficit in the ability to perform a spatial task, as indicated by the effects on the chickens' working memory. Exposure to varying degrees of early environmental complexity thus influences how well birds remember the type of stimulus presented, when it was presented, and where this happened. This deficit may prevent cage-reared hens from finding and obtaining resources in a complex aviary setting (Gunnarsson et al., 2000; Brantsæter et al., 2016a). Despite the behavioural effects of barren rearing on laying hen memory, we found no support for the hypothesis that varying exposure to environmental complexity during rearing should result in differences in TH staining in the hippocampus and NCL (paper **III**). However, as previously mentioned, it is probable that an environment more complex than an aviary, such as a free-range system, could produce the expected differences in TH in laying hens (Patzke et al., 2009). Nevertheless, the proximate effect of aviary versus cage rearing on laying hen memory still needs to be identified.

In conclusion, this thesis demonstrates that the environment experienced during rearing can have considerable, both short and long lasting, effects on the behavioural development of laying hens and on how well they will cope with being housed in the planned adult environment. This, in turn, carries pertinent welfare consequences.

5.5   Some future research needs

As pointed out in paper **III**, dopamine is the not the sole modulator of cognitive functions and there are several other mechanisms of memory formation that could be studied relative to differences in early environmental complexity. A previous study found that adult hens in a free-

range housing system had larger hippocampal cell somas compared to hens housed in conventional cages (Patzke et al., 2009). Studies with 16-day-old chicks found that those reared with visual barriers had better spatial memory (Freire et al., 2004) and longer dendrites with more dendritic spines (Freire and Cheng, 2004) compared to chicks reared without any barriers. Furthermore, memory formation and learning have been shown to be mediated by synaptic plasticity, long-term potentiation (LTP), and the receptors that regulate it (Morris et al., 2003). LTP, the long-lasting increase in synaptic efficiency induced by high-frequency stimulation, is dependent on NMDA receptors (Martin et al., 2000). The use of NMDA receptor antagonists (Morris et al., 1986) or NMDA knockout (McHugh et al., 1996; Tsien et al., 1996) causes deficits in spatial memory. Therefore, it is possible that the effects of rearing environment on working memory seen in paper **II** were due to other changes such as cell soma size, the NMDA receptors, and/or the LTP cascade rather than dopaminergic changes.

One of the results of this thesis work is that provision of substrate during early rearing reduces the likelihood of plumage damage in the production period compared to flocks that did not have access to litter from so early in life. However, flocks from the paper substrate treatment groups still had an average of 10% of the flock with damage score 1 and 2. None of the aviary rearing farms visited during recruitment for paper **IV** provided any form of environmental enrichment, beyond those that supply paper substrate. Further research, therefore, could investigate the effects of more substantial environmental enrichment during rearing on the incidence of feather pecking during the laying period. As mentioned before, the farmers often do not provide any litter material or enrichment and rely on the accumulation of droppings, dust, and feathers from the birds themselves to create a layer of substrate in which the birds can forage and explore. There is evidence for the positive effects proper litter and environmental enrichment have on laying hen welfare due to the reduction of feather pecking, feather damage, cannibalism, and mortality (Blokhuis and Van Der Haar, 1989; Blokhuis and Haar, 1992; Johnsen et al., 1998; Gunnarsson et al., 1999; Jones and Carmichael, 1999b; Jones et al., 2002; McAdie et al., 2005). Despite this extensive knowledge, this is not practised by rearing farmers, perhaps because the majority of these abnormal behaviours occur not during rearing, but during the laying period. The potential detrimental effects of not providing litter in the first weeks of life are therefore not directly seen by the rearing farmers, and thus for this part of the chain, the welfare benefits as seen by the farmer do not weigh up against

the economic costs in terms of labour and materials. A large-scale on-farm study, like that of paper **IV**, on the effects of extensive litter and environmental enrichment provision on the development of feather pecking, would be beneficial in further advocating for this practice.

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Appendices I - IV