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Effects of the rearing period and the production phase on fear-related responses in adult laying hens

Oppdretts- og produksjonsforholds betydning for fryktrelaterte responser hos verpehøns

Margrethe Brantsæter

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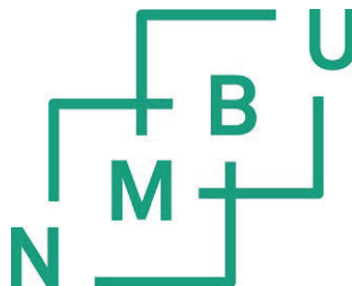
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Summary

In 2013, almost seven billion laying hens (*Gallus gallus domesticus*) produced eggs for human consumption (CIWF, 2013). Under commercial egg production, specialised rearing farmers keep the birds until they are nearly sexually mature at 16–18 weeks of age. Prior to the onset of lay, the birds are transferred to an egg production farm. The housing systems used in commercial rearing and egg production farms are confined systems (cages) and loose-housed systems (single-tier floor or multi-tier aviary systems) (Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015). One of the main differences between these housing systems is the level of environmental complexity. In Norway, the majority of laying hens are reared in aviaries. As a consequence, some of the aviary-reared birds are transferred to furnished cages for egg production. The literature suggests that the environment during early life affects physical and behavioural development. However, little knowledge exists on how aviary rearing, as opposed to cage rearing, affects fear responses in adult laying hens.

Fearfulness is the predisposition of an individual to be easily frightened (Boissy, 1995; Jones, 1996). Fear is the emotion experienced by an individual when exposed to dangerous stimuli. Fear results in behavioural, physiological, and cognitive responses. The conscious component of fear cannot be assessed verbally in animals, but measures of neural, physiological, and behavioural responses can provide information about their emotional state. In the wild, fearfulness is adaptive as it increases the chance of survival. Although fearfulness has been reduced after generations of domestication, predator-avoidance behaviour is still observed in laying hens exposed to humans or novelty (Boissy, 1995; Jones, 1996; Waiblinger et al., 2006). In commercial egg production, high levels of fearfulness and subsequent fear responses are associated with problems such as feather pecking (Uitdehaag et al., 2009; de Haas et al., 2010; de Haas et al., 2013; Kops et al., 2013; Rodenburg et al., 2013), cannibalism (Newberry, 2004) and panic induced smothering (Mills and Faure, 1990; Boissy, 1995; Bright and Johnson, 2011; Richards et al., 2012; Barrett et al., 2014).

The core welfare aspect of this thesis was to investigate the effects of different levels of environmental complexity during the rearing period on fear responses in adult laying hens. Papers **I** and **II** compared the effects of aviary versus cage rearing in birds after transfer from

the rearing farm to the egg production farm. Fear responses were assessed at 19, 21, and 23 weeks of age, tested in the home cage (paper **I**) or in a test arena (paper **II**). The results from papers **I** and **II** suggest that aviary rearing reduces the expression of fear responses compared to rearing in cages. Paper **III** investigated whether the provision of a pecking substrate (a thin layer of paper covering the wire mesh floor) from the first day of life affected fear responses in aviary-reared birds. The control group was reared on the wire mesh, without access to a pecking substrate until later in the rearing period. Fear tests were conducted at the egg production farms when the birds were at the peak of lay. The results from paper **III** indicate that access to a pecking substrate during the early rearing period reduces the fear response to a novel object. However, the effect was only evident in birds that were not provided with environmental enrichment at the egg production farm. In other words, provision of environmental enrichment at the egg production farm can counteract a lack of a pecking substrate during the early rearing period. The results of paper **III** therefore emphasise that both the environmental complexity during rearing and the environment and management during the egg production phase affects fear responses in laying hens. In paper **IV**, risk factors associated with problem behaviours in adult hens were evaluated. The risk factors identified were related to management and housing systems at the production farm. Combined, the results from papers **III** and **IV** emphasise that both the rearing period and the conditions at the production farm affect problem behaviours in adult laying hens.

The complete elimination of fear is neither desirable nor required to safeguard the best welfare possible for laying hens in commercial egg production. However, by including fearfulness in the breeding programs and focusing on optimising environment and management during rearing and production, problem behaviours can be reduced. By increasing environmental complexity and the level of stimulation the chicks and pullets experience during rearing, and by providing environmental enrichment during the production phase, farmers can improve the welfare of commercial laying hens.

Sammendrag

På verdensbasis var det i 2013 nesten 7 milliarder verpehøns (*Gallus gallus domesticus*) som produserte egg til humant konsum (CIWF, 2013). I kommersiell eggproduksjon er unghønene hos spesialiserte oppdrettere fram til dyrene er 16-18 uker gamle. Før hønene har begynt å verpe flyttes de til en eggprodusent. Driftssystemene som brukes i kommersiell eggproduksjon er innredede bur (miljøinnredning) eller frittgående systemer. Frittgående systemer kan være enetasjes gulvanlegg eller fleretasjes aviarier (Erhvervsfjerkræsektionen, 2015). Disse driftssystemene utsetter hønene for forskjellige miljø, særlig med hensyn på kompleksitet. I aviarier kan dyrene bevege seg fritt i ulike høyder og langs bakken. Dyrene får dermed utført viktige atferder som å fly, å flakse med vingene uten å støte borti andre høner eller innredning, sandbading, de har muligheten til å vagle seg i ulike høyder og til å sysselsette seg med å søke etter fôr. Muligheten for å utføre disse atferdene er derimot mer begrenset når dyrene holdes i bur. Miljøet dyrene lever i er viktig for dyrets fysiologiske og mentale utvikling, og dermed dyrevelferden. I Norge blir de fleste verpehøns (80 %) oppdrettet i aviarier, uavhengig av om de skal sendes til eggprodusent med aviar eller miljøinnredning. Det er lite kunnskap om hvordan flytting av dyr fra oppdrettsaviar til miljøinnredning hos eggprodusent påvirker dyrenes fryktresponser.

Fryktsomhet er dyrets tendens til å bli skremt (Boissy, 1995; Jones, 1996). Frykt er emosjonen et individ opplever når det blir utsatt for truende situasjoner. Frykt medfører endring av fysiologi, kognisjon og adferd. Den subjektive opplevelsen av frykt kan ikke undersøkes verbalt hos dyr, men målinger av nevrologiske, fysiologiske responser og observasjon av dyrets adferd kan brukes som indikatorer på emosjonen. I naturen er fryktsomhet gunstig da det øker mulighetene for overlevelse. Etter år med spesifikk avl av verpehøns har fryktsomhet blitt redusert noe. Men det er likevel et problem at verpehøns oppfatter mennesker, ukjente situasjoner og lyder som skremmende (Boissy, 1995; Jones, 1996; Waiblinger et al., 2006). Fryktrelaterte reaksjoner er forbundet med mange problemer i kommersiell eggproduksjon. Fjørhakking (Uitdehaag et al., 2009; de Haas et al., 2010; de Haas et al., 2013; Kops et al., 2013; Rodenburg et al., 2013), kannibalisme (Newberry, 2004) og klumping (Mills and Faure, 1990; Boissy, 1995; Bright and Johnson, 2011; Richards et al., 2012; Barrett et al., 2014) er eksempler på frykt-relaterte atferder som skaper problemer blant verpehøns.

Hovedformålet med denne avhandlingen var å undersøke hvordan graden av kompleksitet i oppdrettsperioden påvirker fryktresponsen hos verpehøns etter de var flyttet til eggprodusent. Artikkel I og II sammenliknet fryktresponser hos høner som var oppdrettet i bur eller i aviar. Fryktrespons ble testet ved 19, 21 og 23 ukers alder enten i hjemmemiljøet (artikkel I) eller i en testarena (artikkel II). Resultatene fra artikkel I og II antyder at aviaroppdrett reduserer dyrenes fryktresponser sammenliknet med dyr som er fra buroppdrett. Artikkel III undersøkte om tilgang på hakkesubstrat (et tynt papirlag oppå nettingen) fra første levedag påvirket fryktresponsen hos aviaroppdrettede fugler. Kontrollgruppen var fugler som ble oppdrettet på netting og dermed ikke fikk tilgang på hakkesubstrat før senere i oppdrettsperioden. Fuglenes fryktrespons ble testet hos eggprodusentene på et tidspunkt hvor dyrene hadde nådd verpetopp. Resultatene fra artikkel III tyder på at tilgang på hakkesubstrat reduserte dyrenes frykt når de ble utsatt for en ukjent gjenstand. Den fryktreduserende effekten gjaldt kun dersom dyrene ikke hadde tilgang på miljøberikende elementer hos eggprodusenten. Med andre ord: dersom oppdretteren ikke gir dyrene tilgang på hakkesubstrat kan eggprodusenten redusere dyrenes fryktsomhet ved å gi dem miljøberikelse. Dette resultatet indikerer at forhold både i oppdrettsperioden og hos eggprodusent er viktig for dyrenes fryktrespons. Artikkel IV hadde som mål å undersøke risikofaktorer i oppdrettsperioden og hos eggprodusent som hadde sammenheng med forekomst av problematferder. De fleste av disse problematferdene kan sees i sammenheng med frykt. Faktorene som ble identifisert i artikkel IV var relatert til innredningstypen og rutiner hos eggprodusenten. Hvis man ser på kombinasjonen av resultatene fra artikkel III og IV, tydeliggjør de at det ikke bare er oppdrettsperioden, men også forhold hos eggprodusenten er viktig for fryktrelaterte problematferder hos verpehøns.

Å fjerne frykt fullstendig er verken ønsket eller nødvendig for å sikre best mulig dyrevelferd hos verpehøns. Hvis fryktsomhet kan inkluderes i avlsarbeidet og man fokuserer på å optimalisere miljøet både i oppdrettsperioden og hos eggprodusent kan forekomsten av fryktrelaterte problemer minskes. Ved å øke variasjonen i stimuli dyrene blir utsatt for, og ved å supplere med miljøberikelse kan dyrevelferden hos verpehøns i Norge forbedres.

Abbreviations

ANOVA = analysis of variance

AV = aviary-reared

C = cage-reared

EIA = enzyme immunoassay

FCM = faecal corticosterone metabolites

HAT = human approach test

HPA = hypothalamic–pituitary–adrenocortical

NOT = novel object test

OR = odds ratio

PCA = principal component analysis

SPT = stationary person test

TI = tonic immobility

WQ = Welfare Quality

List of papers

Paper I

Rearing laying hens in aviaries reduces fearfulness following transfer to furnished cages.

Brantsæter M, Tahamtani FM, Moe RO, Hansen TB, Orritt R, Nicol C, Janczak
AM. 2016. Frontiers in Veterinary Science 3 DOI: 10.3389/fvets.2016.00013

Paper II

Exposure to increased environmental complexity during rearing reduces fearfulness and increases use of three-dimensional space in laying hens (*Gallus gallus domesticus*).

Brantsæter M, Nordgreen J, Rodenburg TB, Tahamtani FM, Popova A, Janczak
AM. 2016. Frontiers in Veterinary Science 3 DOI: 10.3389/fvets.2016.00014

Paper III

Access to litter during rearing and environmental enrichment during production reduce fearfulness in adult laying hens.

Brantsæter, M, Tahamtani, FM, Nordgreen, J, Sandberg, E., Hansen, TB, Rodenburg, TB,
Moe, RO, Janczak, AM. 2016. Applied Animal Behaviour Science DOI:
10.1016/j.applanim.2017.01.008

Paper IV

Problem behaviours in adult laying hens – Identifying risk factors during rearing and egg production

Brantsæter M, Nordgreen J, Hansen TB, Muri K, Nødtvedt A, Moe RO, Janczak AM. 2017.
In press. Poultry Science.

1. Introduction

The increased demand for proteins for human consumption has caused a shift from small-scale backyard egg farming to intensive, specialised egg production (Cornish et al., 2016). In 2013, almost 7 billion laying hens (*Gallus gallus domesticus*) produced eggs for human consumption (CIWF, 2013). The large number of laying hens emphasises the importance of further developing our understanding of laying hen welfare under intensive farming conditions. The environments hens would experience in the wild differ markedly from the conditions in commercialised egg production. In the wild, the Red Junglefowl (*Gallus gallus*) lives in stable social groups (Väisänen et al., 2005) and spend the majority of its time searching for feed (Schütz and Jensen, 2001). Egg laying only happens during late spring, and the hens lay 10–15 eggs per year (McBride et al., 1969). Laying hens kept for egg production, on the other hand, are bred to produce 325 eggs per year; they live in groups much larger than in the wild, and their ability to perform innate behaviours is largely dependent on the system in which they are housed.

Growing public concern over animal welfare questions and increased research documenting laying hens' strong motivations to dust bathe, to lay eggs in secluded nest boxes, to perch and to perform foraging behaviour, resulted in the EU ban on battery cages in 2012 (European Commission, 1999). As opposed to the barren battery cages where the birds only have access to feed and water, the alternative housing systems (furnished cages and aviary systems) accommodate a wider range of the laying hens' needs. Norwegian national legislation states that birds destined to produce in loose-housed systems must have access to litter during the rearing period (Landbruks- og matdepartementet, 2001). However, there are no commercial rearing cages on the market that fulfil this requirement. This means that in Norway the majority of hens are reared in aviaries and sent to egg producers with either furnished cages or aviaries (Animalia, 2016). Previous studies have reported detrimental effects on animal welfare in aviary-reared birds after transfer to furnished cages (Tahamtani et al., 2014). However, the effect on fear responses associated with being reared in an aviary and moved to furnished cages for egg production is unknown.

The core welfare aspect of this thesis was to investigate the effects of aviary rearing compared to cage rearing on fear responses after the onset of lay. Also, providing access to a pecking

substrate from the first day of life was tested as a potential method to reduce fear responses in adult hens under commercial egg production. Furthermore, risk factors associated with problem behaviours during the rearing and production phase were identified.

1.1. Egg production worldwide

Asia is the continent with the greatest egg production (Bagley, 2016b). China is the leading egg producing country with 1.2 billion laying hens reported in 2014 (Erhvervsfjerkræsektionen, 2015). The other top egg producing countries from 2014 were the USA (305 million laying hens), India (206 million laying hens), Mexico (152 million laying hens) and Japan (134 million laying hens) (Erhvervsfjerkræsektionen, 2015; Bagley, 2016b). The total number of laying hens within the European Union was roughly the same as in the USA, with 363 million laying hens in 2011 (CIWF, 2013). The top three egg producing countries in the EU in 2014 were France, Spain, and Germany (Erhvervsfjerkræsektionen, 2015).

Worldwide, there are large differences with regards to the housing systems in use. Globally, around 60% of laying hens are housed in battery cages (European Commission, 2011). In a battery cage, birds are confined within a barren environment including only feed, water, and a few other conspecifics. However, research has documented that battery cages prevent laying hens from expressing behaviours they are strongly motivated to perform (Duncan, 1998). As of 2012, within Europe, 'all hens must have a nest, perching space, litter and unrestricted access to a feed trough' (EU directive 1999/74/EC) (European Commission, 1999). The ban caused a shift towards housing in single-tier (barns) and multi-tier (aviaries) systems, with or without access to outdoor areas. In 2014, approximately 50% of the layers in the EU member states were housed in loose-housed systems (EC-CIRCABC, 2014).

1.2. The Norwegian egg industry

In 2015, the Norwegian Agricultural Authorities had registered 16 commercial rearing farmers and 585 egg producers with flocks of at least 1,000 birds (Bagley, 2016b). The total number of laying hens in Norway at any given time in 2016 was 4.2 million (Animalia, 2016). Norwegian egg production relies on imported hybrids. In 2015, 70% of the laying hens in Norwegian egg production were Lohmann Selected Leghorn (Lohmann Tierzucht, Germany) and 30% were

ISA Dekalb White (ISA Hendrix Genetics, The Netherlands) (Christoffer Singstad, *personal communication*, April 2017). One consequence of import is that the Norwegian poultry industry has little if any influence on the genetic characteristics the companies emphasise in their breeding programs. The majority (97%–98%) of hens used in commercial egg production lay white eggs while the remaining 2%–3% lay brown eggs (NFL, 2017). Compared to brown strains, white laying hens have been found to be more fearful in tonic immobility tests (Albentosa et al., 2003) and to have a higher plasma corticosterone response after exposure to stressors (Fraisse and Cockrem, 2006). White layers are also more hesitant to approach novel objects and humans (Oden et al., 2002; de Haas et al., 2013). In other words, problems associated with fear responses are likely to be a relevant concern in the Norwegian egg production.

The health status of Norwegian laying hen flocks is exceptional. The only two vaccines routinely administered are an injected vaccination against Marek's disease immediately after hatching, and a coccidiosis vaccine sprayed on the feed early in the rearing period (Griffiths, 2016). No other country is comparable regarding the limited use of vaccines and antibiotics (Bagley, 2016b).

In addition to legislation controlled by the European Union, the Norwegian egg industry has to follow strict national regulations. Beak trimming has been banned since 1974 (Frøslien, 1997) in contrast to the majority of the rest of the world where flocks are still beak trimmed. Also, the maximum number of hens allowed per egg production farm is 7,500 birds (Landbruks- og matdepartementet, 2004). Norwegian laying hen flocks are thus small compared to those in other European countries such as Sweden (average of 23,000 birds per farm) (Svenska ägg, 2015) and Belgium (average > 30,000 birds per farm) (Tuyttens et al., 2011; Heerkens et al., 2016a).

Norway is among the few countries worldwide in which the majority of adult laying hens are housed in loose-housed systems (Erhvervsfjerkræsektionen, 2015). In 2016, 58% of adult laying hens were housed in aviary systems, 36% in furnished cages, and 6% of flocks produced eggs under organic conditions (Animalia, 2016). The Norwegian legislation specifically mentions that all birds must have access to litter and perches during rearing (Landbruks- og matdepartementet, 2001). However, no rearing cages on the market fulfil the requirement of access to litter. Until April 2014, rearing farmers were given temporary permission to rear birds

in traditional barren cages. However, since the extension has expired, most laying hens in Norway (80%) are reared in aviaries (NFL, 2016). The rearing farmers that wanted to rear birds destined to produce in furnished cages had to add perches and a litter area to their barren rearing cages (Nils Steinsland 15 February 2017, *personal communication*). A consequence of the high number of birds reared in aviaries is that a proportion of aviary-reared birds are transferred to furnished cages and thus experience a change in housing system from the rearing period to the production phase.

Lastly, Norway's topography and geographical location give rise to large local and seasonal variations, particularly regarding temperature. As a consequence, designing hen houses and appropriate systems for ventilation and air quality control can be challenging. Issues with climatic control such as draught or uneven temperatures can result in aggregation of birds (increased stocking density) in parts of the house. There is limited knowledge on effects of stocking density on welfare in loose-housed hens (Widowski et al., 2016). Some, but not all studies, indicate negative consequences for welfare at increased stocking densities (Channing et al., 2001; Mashaly et al., 2004; Nicol et al., 2006). Also, areas with clustering, or low ventilation can cause 'blind spots' where gases such as ammonia and carbon dioxide build up. Ammonia can cause health problems not only among the animals but also for the stock people (Kirkhorn and Schenker, 2002; Kirychuk et al., 2003; David et al., 2015a; David et al., 2015b). The threshold of maximum exposure of 25 ppm of ammonia is decided based on human safety but exceeds the preferred ammonia concentrations as assessed in preference tests of adult hens (Kristensen et al., 2000). If the temperature is too low, the birds have to increase the feed intake to keep warm (in the winter), which is both a stressor for the animals and costly for the farmer. Low air humidity can make feathers brittle, which can increase heat loss and increase the risk of feather pecking (Bagley and Rædergård, 2016). To date, limited data exists about the extent of issues with climatic conditions in laying hen production farms in Norway.

The structure of the Norwegian egg industry is strictly hierarchical (Figure 1). This structure allows for good control of genetic material as well as prevention and control of disease (Bagley, 2016a). The following sections will highlight some of the important aspects of the egg production chain, emphasising some of the fear-related challenges in Norwegian egg production.
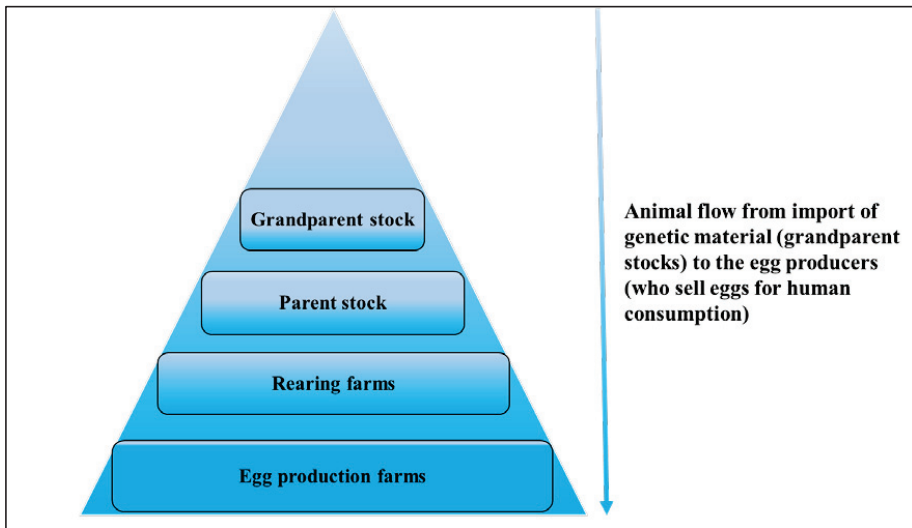
Figure 1. Schematic drawing illustrating the organisation of the Norwegian egg production system (based on text in (Bagley, 2016a). Grandparent stock is imported. The offspring from the grandparent stock found the parent stock, which lay eggs that are hatched and transferred to specialised rearing farms. The hens remain at the rearing farm until 15–16 weeks of age prior to being moved to the egg production farm, where they produce eggs until euthanised at 70–80 weeks of age. Illustration: Margrethe Brantsæter.

1.2.1. Genetic material

All genetic material is imported as one-day-old chicks. Lohmann chicks are imported from the breeding company Lohmann Tierzucht in Cuxhaven, Germany, while Dekalb White and ISA Brown chicks come from ISA Hendrix Genetics, the Netherlands. These animals comprise the grandparent stock (Figure 1). The grandparent stock produce eggs resulting in parent stock. To ensure fertilised eggs, about 8% of the birds in the grandparent and parent flocks are roosters. The majority of grandparent stock and parent stock are housed in aviaries, and a minority are housed in single-tier floor systems (Bagley, 2016a). Fertilised eggs from the parent stock are collected several times per day and sent to hatcheries. In 2016, there were two commercial hatcheries in Norway, one for Lohmann laying hens and one for Dekalb and ISA brown layers. The hatching process is strictly controlled with regards to handling, temperature, air humidity, air circulation, and gas concentrations. The incubation is optimised to ensure that the eggs hatch in synchrony after 21 days (Bagley, 2016a). After hatching, the male chicks are euthanised, while the female chicks are injected with the Marek's vaccine and transported to one of the 16 commercial rearing farms nationwide.

1.2.2. Early life effects on behavioural development

Behavioural characteristics of an individual are determined by its genes, and by prenatal and early life environment (Rodenburg and de Haas, 2016). During early developmental stages, the chick is sensitive to epigenetic changes (Rodenburg, 2014) and maternal hormones transferred via egg yolk and albumin (Groothuis et al., 2005). Eggs injected with corticosterone resulted in chicks with increased avoidance of humans when tested at 12–14 days of age (Janczak et al., 2006). In addition to the environment inside the egg, exposure to external stimuli (e.g. light, odour, and sounds) affects ontogeny (Rogers, 1995; Rogers, 2008; Bateson et al., 2014). Prenatal and early life cues help the organism to prepare for the environment encountered later in life (Bateson et al., 2014). The work presented in this thesis focuses on how the early environment affects fear responses in adult hens. Soon after hatching, a series of different behaviours start to develop. Chicks imprint on conspecifics (Bateson, 1966; Nordgreen et al., 2006) and start to avoid and show fear responses when exposed to novel stimuli within 48 hours after hatching (Sluckin and Salzen, 1961). In nature, as opposed to under commercial rearing conditions, chicks are provided with maternal care. The presence of maternal care results in epigenetic changes that reduce fear responses in mice (Curley et al., 2008) and anxiety and cannibalistic pecking in laying hens (Riber et al., 2007; Rodenburg et al., 2009). Perching behaviour starts to develop in the first week of life (Heikkilä et al., 2006). Birds deprived of perches during rearing had increased duration of tonic immobility at 15 and 20 weeks of age compared to birds reared with access to perches (Brake et al., 1994). These studies indicate that the early environment is relevant for chicks' development of fear responses. The complete elimination of fear is neither desirable nor required to safeguard the best welfare possible for laying hens in commercial egg production. However, preventing a mismatch between the rearing and production environments might reduce the impact of problems related to exaggerated or inappropriate fear responses.

The rearing period should prepare the chicks physically and behaviourally for the environment they will experience during the laying phase (Rogers, 1995; Rodenburg et al., 2008a; Janczak and Riber, 2015). Specialised rearing farmers take care of the pullets from when they arrive as one-day-old chicks until they are transferred to the egg production farm before the onset of lay. As the majority of laying hens in Norway are transferred to loose-housed systems as adults, most farmers have installed rearing aviaries. The rearing aviaries lack nest boxes, the group size is bigger, and the stocking density is higher compared to the egg production aviaries. A

typical aviary rearing farm in Norway contains at least 15,000 birds. This is twice the maximum number of birds allowed at the egg production farms (Landbruks- og matdepartementet, 2004). Furthermore, the maximum stocking density allowed is 24 birds per m^2 when the chicks are 5– 16 weeks and maximum 22 animals per m^2 after 16 weeks of age (Landbruks- og matdepartementet, 2001). During egg production, the maximum stocking density allowed is nine birds per m^2 (Landbruks- og matdepartementet, 2001) (Photo 1). Provision of feed and water occurs in the aviary rows, and the pullets have access to perches at different heights (Photo 2). However, it may be physically impossible for the chicks to move between the rows and corridors of the aviary until they reach a certain size. To prevent them from starving, the chicks are therefore not released from the aviary rows until they are about five weeks of age and can move back up to the feed and water (Photo 3). If the rearing farmer knows that the chicks will be sent to an egg producer with furnished cages, they can simulate cage rearing by never opening the doors of the aviary row (Photo 3). In this case, the birds will never experience the full three-dimensional space of the aviary system and the level of environmental complexity will be limited compared to rearing in the entire aviary-system.

Photo 1 illustrates a rearing aviary with birds of 11 weeks of age. Legislation states that the maximum stocking density allowed until 16 weeks of age is 24 birds per m^2. Photo credit: Fernanda M. Tahamtani.

Photo 2 shows the inside of an aviary row (rearing aviary). The white manure belt is visible below the wire mesh floor. The feed trough separates the front (the right side of the picture) and the back (the left side of the picture) of the row. Above the feed trough, the birds can perch on the metal pipe. The white and red drinking nipples are visible along the back wall (left side in the picture). Photo credit: Margrethe Brantsæter

Photo 3 shows birds approximately 11 days old, confined inside the aviary rows. The birds will be allowed to enter the corridor (the right side of the picture) when they are big enough (5–6 weeks old) to climb back up to access feed and water. Perches (attached along the isles of the aviary row) will be made available when the birds are released from the aviary rows. If the rearing farmer knows the birds will be housed in furnished cages during production, he or she can keep the birds confined inside the aviary row for the entire rearing period. Photo credit: Fernanda M. Tahamtani.

Previous studies have shown that the level of environmental complexity affects long-term cognitive abilities (Gunnarsson et al., 2000; Tahamtani et al., 2015). However, limited research has focused on the effect of environmental complexity during rearing on fear responses in adult laying hens. The few studies that have been conducted confound the environment during rearing and later production (Brake et al., 1994; Colson et al., 2006) and have only compared cage rearing with floor rearing, rather than with aviary rearing (Anderson and Adams, 1994; Johnsen et al., 1998).

Some of the routines implemented by the rearing farmers differ. For instance, the age at which the birds are released into the aviary corridors, whether they add sawdust or other litter on the ground before they open the doors of the aviary rows, and the amount of time they spend inspecting the pullets. However, one aspect of management with potential welfare consequences is the routine followed while the pullets are enclosed inside the aviary rows. Some rearing farmers cover the wire mesh inside the aviary row with paper and sprinkle sparse amounts of feed on top to help the birds access feed easily upon arrival. In addition, particles of dust and faeces accumulate on the paper and thus form a pecking substrate. Other rearing farmers leave the wire mesh uncovered, resulting in the pullets getting access to a pecking substrate only after they are released into the aviary rows at 5–6 weeks of age (Photos 4–6). Previous studies have found that early access to litter reduces feather pecking and improves plumage condition in adult hens (Huber-Eicher and Wechler, 1998; de Jong et al., 2013; de Haas et al., 2014a; Tahamtani et al., 2016a). One study assessed the birds' willingness to approach a stationary person. They found that litter disruption during rearing increased fear of humans at 40 weeks of age (de Haas et al., 2014a). These results are not necessarily comparable to those of Norwegian conditions, as the hens were beak trimmed, which can influence behaviour and thus study results (Davis et al., 2004; Janczak and Riber, 2015).
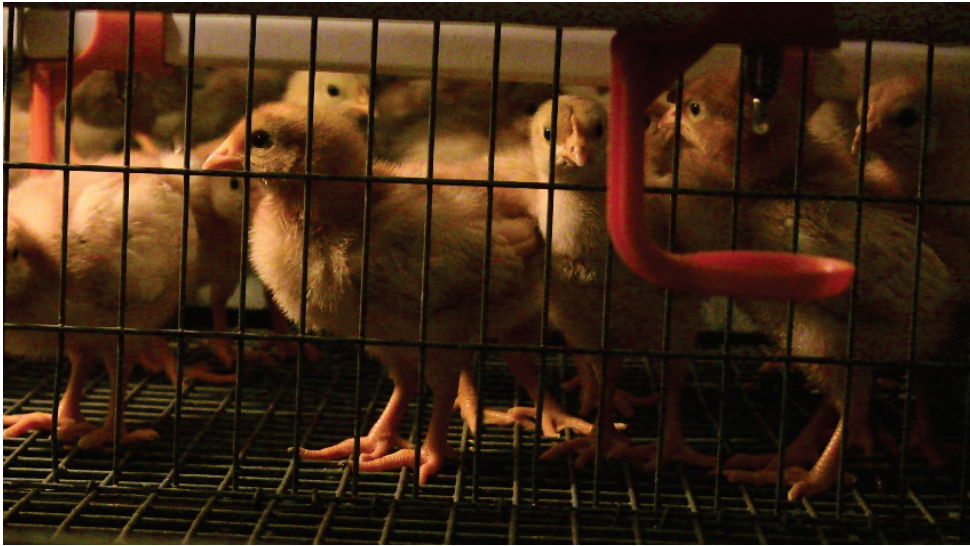
Photo 4 illustrates chicks about four days old confined inside the aviary row walking on the wire mesh. These chicks will not have access to a pecking substrate until they are released into the corridors of the aviary at 5–6 weeks of age. Photo credit: Fernanda M. Tahamtani.

Photos 5 and 6 show an aviary row where the wire mesh is covered with a layer of paper, before arrival (photo 5) and a few days after arrival (photo 6). The paper allows accumulation of feed, dust, and faeces and constitutes a pecking substrate for the chicks from day one of the rearing period. Photo credit: Tore Villanger.

1.2.3. The production period

The birds are transported from the rearing farm to the egg producer at 15–18 weeks of age. A recent review recommends that birds should be transported before they are 16 weeks of age (Janczak and Riber, 2015) as this allows them to adjust to their new environment before the onset of lay. The housing system mainly determines the environmental conditions and management during lay. Environmental enrichment is the other major influential factor. The

term 'environmental enrichment' is defined in this thesis as any supplement in addition to feed or water that encourages active, explorative, and foraging behaviours. Examples of environmental enrichment supplied by farmers are empty plastic boxes, box lids, toy balls, old CDs, pecking stones (aerated concrete and calcium silicate hydrate blocks), sawdust, oyster shells, and cut up pieces of manure belts or egg belts. Under experimental settings, Nicol et al. (2001) found that adult birds with access to straw performed significantly more ground pecking and less feather pecking compared to birds housed without access to straw, regardless of experience during rearing (Nicol et al., 2001). In addition to reducing feather pecking (Jones, 2002; McAdie et al., 2005; Rodenburg et al., 2013) and cannibalism (Newberry, 2004), environmental enrichment might reduce fear responses in adult laying hens (Jones, 1996; 2002; 2004). In other words, the behaviour expressed by an individual is not only dependent on the rearing period, but also on the environment during adulthood (Nicol et al., 2001; Hartcher et al., 2016). However, only anecdotal information exists about the effects of enrichment under commercial egg production in Norway.

From an animal welfare perspective, the main differences between loose housing systems and confined systems are group size, freedom of movement, and environmental complexity (reviewed by e.g. EFSA, 2005; Rodenburg et al., 2005; Lay et al., 2011). The following paragraphs will briefly discuss the different housing systems, with emphasis on environmental complexity and the birds' ability to express natural behaviours, both positively and negatively, in a welfare context.

1.2.3.1. Confined housing systems: furnished cages

Confined housing systems are either furnished cages (maximum ten birds per cage) or colony cages (up to 100 birds per cage) (Rodenburg et al., 2005). Of the 36% of egg farms with confined housing systems in Norway, the majority keep the birds in furnished cages, with a maximum of nine hens per cage (Bagley and Rædergård, 2016).
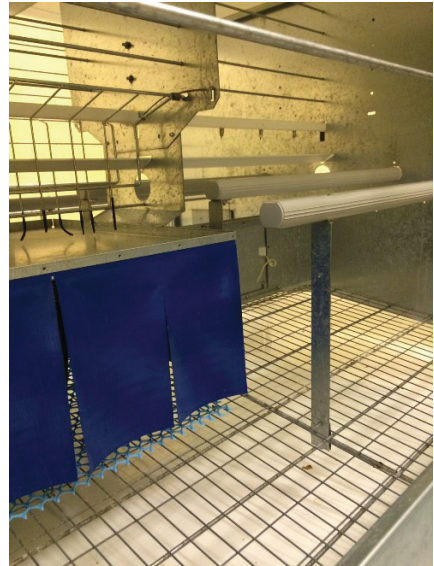
Photos 7 and 8 show the furnished cages at the experimental facilities at NMBU, Adamstuen. Each furnished cage contains a nest box (blue plastic covers the front of the nest box), a designated dustbathing area (on top of the nest box) and two perches. Water pipes with drinking nipples along the back wall are visible in the right half of the cages. The feed trough runs along the front of the furnished cages. The wire mesh floor is slightly tilted to ensure eggs roll down onto the egg collection belt below the feed trough. The claw trimmer, situated on the inside of the feed trough, is hidden from view. Photo credit: Margrethe Brantsæter.

The legislation requires furnished cages to contain nest boxes, perches, a claw trimmer, and litter, in addition to an *ad libitum* supply of feed and water (Landbruks- og matdepartementet, 2001) (Photos 7 and 8). Compared to the battery cages, these resources allow the birds to satisfy more of their behavioural needs. However, the confined space limits the environmental complexity and restricts the birds' exposure to stimuli. Regarding the stocking density, the European directive states that each hen should have at least 750 cm^2 of cage area (European Commission, 1999). The Norwegian legislation is more stringent and dictates that each hen should have access to at least 850 cm^2 of cage area (Landbruks- og matdepartementet, 2001).

If we compare welfare in different housing systems, birds housed in furnished cages often have lower mortality rates and lower prevalence of disease due to less contact with faeces and better air quality (EFSA, 2005; Rodenburg et al., 2008b; Fossum et al., 2009; Jansson et al., 2010; Lay et al., 2011; David et al., 2015a; David et al., 2015b). From a biological perspective, this could be interpreted as furnished cages ensuring better animal welfare. However, in furnished cages, laying hens have limited space in which to dustbathe, flap and stretch their wings, and perform foraging behaviour (Widowski et al., 2016). Studies report a lower occurrence of

dustbathing and higher occurrence of sham dustbathing in furnished cages compared to loose-housed birds (Olsson and Keeling, 2005). For example, lack of dustbathing and increased occurrence of sham dustbathing in cage-housed hens compared to birds housed in aviary systems (Vestergaard et al., 1997; Cooper et al., 2004) may be indications of higher levels of stress in cage-housed hens. Early access to dustbathing material and the type of dustbathing material provided have been found to be crucial for the occurrence of dustbathing in furnished cages (Olsson et al., 2002). To date, we have only anecdotal information based on oral communication with farmers on how furnished cages are managed under Norwegian conditions.

Any environmental change an animal experiences can cause stress that compromises its welfare. This issue is particularly relevant for aviary-reared birds that are transferred to furnished cages. Aviary-reared birds were found to show less alert behaviour at 19 and 21 weeks of age and had higher mortality rates throughout the production period compared to cage-reared birds after transfer to furnished cages (Tahamtani et al., 2014). However, few studies have focused on effects of aviary rearing compared to cage rearing on birds' fear responses after transfer to a more confined housing system.

1.2.3.2. Loose-housed systems: single-tier (barn/floor) or multiple-tier (aviary)

In Norway, the majority of loose-housed birds are kept in indoor multi-tier aviary systems (NFL, 2016). Birds in loose-housed systems have more available space than birds in confined systems; the maximum allowed stocking density is nine birds per m^2 (1,111 cm^2 per bird) (Landbruks- og matdepartementet, 2001). The option to move between different heights provides birds in aviaries with more available space and increases environmental complexity compared to housing in single-tier systems. The greater freedom to walk, run, and fly is positive as it strengthens the birds' bone structure (Wilkins et al., 2011). However, it also increases the risk of injuries (e.g. keel bone fractures [Rodenburg et al., 2005; Rodenburg et al., 2008b; Sandilands et al., 2009; Wilkins et al., 2011; Heerkens et al., 2016a; Heerkens et al., 2016b]) or suffocation if too many birds cluster in certain areas of the henhouse (Channing et al., 2001). The high number of conspecifics in loose-housed systems is another factor that increases the stimuli birds are exposed to. The flock size in loose-housed systems exceeds the number of conspecifics (90) a bird can recognise (Väisänen et al., 2005).

For loose-housed systems, Norwegian legislation specifies that at least a third of the floor (equal to 250 cm^2 per bird) should contain litter (Landbruks- og matdepartementet, 2001). This enables the birds to perform highly motivated behaviours such as pecking, scratching and dustbathing (Rodenburg et al., 2005). However, some egg producers have issues with hens laying their eggs in the litter rather than in the designated nest boxes (Bagley and Rædergård, 2016). Mislaid eggs increase the labour required of farmers and reduce their income and are thus mainly a problem for the farmer. However, laying eggs outside the nest boxes can also be a welfare issue as it increases the risk of hens becoming victims of vent pecking (Newberry, 2004). The increased amount of litter in loose-housed systems might lead to poor air quality, which negatively influences the welfare of the farmer (Larsson et al., 1999; Kirkhorn and Schenker, 2002; Kirychuk et al., 2003; Green et al., 2009) and the hens (David et al., 2015a; David et al., 2015b). The extent of issues with climatic conditions in loose-housed systems in Norwegian laying hen flocks is unknown.

1.3. Animal welfare: a brief historical overview

The current view on animal welfare is summarised in the Norwegian Animal Welfare Act as follows: 'animals have intrinsic value, irrespective of the value they have to us as human beings' (Landbruks- og matdepartementet, 2009). However, this has not always been the case. Some of the ancient Greek philosophers (e.g. Aristotle (384–322 BCE)) considered that the only purpose of animals was to serve our human needs. René Descartes (1596–1650) viewed animals as machines that are unable to feel pain or suffering (Wilson, 2016). A substantial contribution to animal welfare science was made by Jeremy Bentham (1748–1832). He was one of the first to claim that animals have the ability to suffer and suggested that it is the ability to suffer, not 'to reason nor think', that makes an animal's welfare relevant and important (Sunstein, 2003).

1.3.1. Public concerns about laying hen welfare

Public concern for farm animal welfare has grown considerably since the beginning of the 21st century (Miele and Lever, 2013; Cornish et al., 2016). The public was first made aware of animal welfare problems related to intensive farm animal production by the book *Animal Machines*, published by Ruth Harrison in 1964 (Harrison, 1964). The book criticised the industrialisation of meat production, and particularly confined housing of chickens, pigs, and

veal calves. Harrison's work marked the beginning of animal welfare science. The British government founded a committee chaired by Professor Roger Brambell in 1965. The subsequent 'Brambell Report' stated that animals should have the freedom 'to stand up, lie down, turn around, groom themselves and stretch their limbs' (Brambell Committee, 1965). The Brambell statement became known as the 'Five Freedoms'. The Five Freedoms were further developed by the UK Farm Animal Welfare Council (FAWC) to include freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury, or disease, freedom to express normal behaviour, and freedom from fear and distress (FAWC, 2009). In other words, according to FAWC, a farming system that fails to meet the requirements of the Five Freedoms implies that animal welfare is compromised. In a 2005 EU survey, only 32% of respondents considered that commercial egg production ensured "good laying hen welfare" (European Commission, 2005). The respondents ranked the condition of laying hens as the worst in terms of animal welfare, followed by the conditions experienced by broilers and pigs. Nonetheless, research indicates that the public has very limited knowledge about animal welfare, particularly in food production (reviewed by Cornish et al., 2016). The public considers captive environments that inhibit or limit innate behaviours as the greatest welfare concern (Widowski et al., 2016). The public's opinion has to be acknowledged as a powerful and influential driving force promoting change in food production and animal welfare legislation. The European ban on battery cages is an example of a change to food production legislation impacted by the public's opinion (Miele and Lever, 2013).

1.3.2. Assessment of animal welfare

Animal welfare is conceptualised as a continuous spectrum ranging from 'very poor' to 'very good' (Fraser et al., 1997; Fraser, 2008; Hemsworth et al., 2015). To scientifically evaluate animal welfare, one has to use empirical methods to inform ethical decision-making regarding animals' quality of life (Widowski et al., 2013). 'Quality of life' is often used to express the sum of an individual's positive and negative experiences (Alrøe et al., 2001). However, a major challenge in this context is that people have different opinions about what constitutes good 'quality of life' and therefore use different definitions and criteria to define 'animal welfare'. The main criteria have been grouped by Fraser et al. (1997) into three broad overlapping categories that form the basis for different approaches to animal welfare science: biological functioning, natural living, and subjective experience (Figure 2) (Fraser et al., 1997; Fraser, 2008).
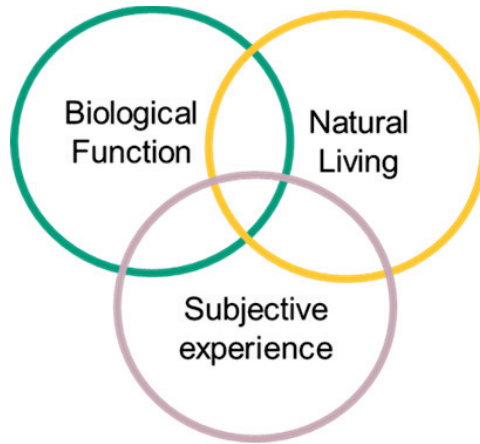
Figure 2 illustrates the components that need to be covered by the definition of animal welfare (based on text in Fraser et al. (1997) and Fraser (2008)). Good animal welfare can only be achieved when the different criteria are covered. Optimal welfare is visualised as the overlap between biological functioning, subjective experience, and natural living. Illustration: Margrethe Brantsæter

The biological function approach considers welfare to be 'good' when the animal succeeds in coping with its environment (Broom, 1986). Coping success involves absence of (large) physiological stress responses and fulfilment of the animal's biological needs (Duncan, 2005). Biological function can be measured objectively as growth, nutritional status, reproductive ability, productivity, and presence/absence of injury or disease (Broom, 1991). The biological function approach, therefore, relies on assessing variables that are easy to measure and quantify. Farmers often express this view of animal welfare: 'As long as my chickens grow well and look healthy, I suppose their welfare is OK' (Te Velde et al., 2002).

The natural living approach emphasises the 'naturalness' of the circumstances the animal experiences and the ability of the animal to live according to its nature (Fraser, 2008). This view of welfare considers access to outdoor areas as crucial and disregards the concurrent increased risks of injury, disease, starvation, and predation (Fraser et al., 1997; Alrøe et al., 2001; Hewson, 2003; Hegelund et al., 2006; Knierim, 2006). This is the view of animal welfare often expressed by the general public (Te Velde et al., 2002; Carenzi and Verga, 2009; Miele and Lever, 2013). Each species has evolved specific physical, mental, and behavioural characteristics to ensure the best possible survival and reproductive potential (Waiblinger et

al., 2004). Consequently, to assess welfare using the natural living approach we need to establish the species-specific needs before establishing what the 'natural' living requirements are (Špinka, 2006; Fraser, 2008).

The subjective experience approach focuses on the affective state of the animal as the core determinant of welfare. The affective state refers to the feelings, moods, or emotions experienced by the animal (Russell, 2003; Walker et al., 2012). Negative affective states such as pain, fear, and frustration are associated with situations of suffering that the animal will avoid if possible (Walker et al., 2012). On the contrary, the animal will be motivated to work for and prefer the pleasurable experience of positive affective states (e.g. comfort and contentment) (Dawkins, 2004; Duncan, 2005; Dawkins, 2006; 2008; Walker et al., 2012; Hemsworth et al., 2015). As one cannot access the subjective state of any individual, assessment of affective states relies on indirect measures such as preference tests and motivational tests. These tests are useful but can be more difficult to interpret than measures acquired from the biological function approach (Duncan, 2005). A more unifying approach, published in 2015, proposed that affective states should be considered an integral part of biological functioning and that affective states can thus be viewed as products of the animals' biological function (Hemsworth et al., 2015).

What one considers the most important welfare criteria is ultimately a value-based decision (Fraser et al., 1997). There is, however, agreement within the scientific community that absence of disease and good health are both required for an animal to have acceptable welfare (Duncan, 2005; Cockram and Hughes, 2011). However, animals might still suffer from poor welfare while in good physical health if they are deprived of activities or resources to which they are highly motivated to have access (Brambell Committee, 1965; Dawkins, 2004; Ladewig, 2008). The best animal welfare can only be achieved when all the criteria are met, as visualised by the overlap in Figure 2, or when the following questions are positively answered: 'Are the animals healthy? Do they have what they want?' (Dawkins, 2004). This thesis uses the definition of animal welfare provided by the World Organisation for Animal Health (OIE): 'Welfare is how an animal is coping with the conditions in which it lives. An animal is in a good state of welfare (as indicated by scientific evidence) if it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress' (OIE, 2016). On the conceptualised scale of welfare, ranging from poor to good, the OIE definition describes a 'neutral' welfare state. For an animal to have 'good' welfare, the

animal should not only be protected from experiencing high levels of fearfulness, but should also be provided with an environment that enables the animal to experience positive feelings. Simply put, not being scared is not the same as being happy.

From the OIE definition, it follows that a welfare assessment should ideally involve a combination of behavioural, physiological, and clinical observations (Dawkins, 2006; Broom, 2011). However, the majority of the work presented in this thesis is based on behavioural indicators of welfare, while measures of stress have only been included to a limited extent. Behavioural responses can provide information on several of the welfare dimensions. For instance, gait scoring provides information not only about physical health (e.g. lameness) but also about affective state (e.g. pain) (Rutherford, 2002; Dawkins, 2004).

1.4. Fear, fearfulness, and fear-related behaviours in laying hens

Because of its adverse effects on the animal's subjective experience and biological function, fear is an important welfare indicator. Fear is the emotion experienced by an individual when exposed to dangerous stimuli (Figure 3). Fear results in behavioural, physiological, and cognitive responses. Fear responses that are exaggerated, inappropriate, or expressed in a restrictive environment can decrease cognitive abilities (Steimer, 2002; Ohl et al., 2008), increase the risk of injuries or mortality, and reduce productivity (Anderson and Adams, 1994; de Haas et al., 2013; Gilani et al., 2013). Fearfulness is the predisposition of an individual to be easily frightened (Boissy, 1995). Fearful individuals do not constantly display fear responses, but, when exposed to fear-inducing stimuli, fearful animals are likely to show more intense or prolonged responses compared with less fearful individuals. The evolutionary purpose of fearfulness is to protect the animal from dangerous situations and consequently increase its chances of survival (Boissy, 1995). However, under commercial farming conditions where most of the dangers birds would encounter in the wild (e.g. predation) are controlled for, high levels of fearfulness are undesirable. In commercial egg production, high levels of fearfulness are associated with problem behaviours such as feather pecking (Uitdehaag et al., 2009; de Haas et al., 2010; de Haas et al., 2013; Kops et al., 2013; Rodenburg et al., 2013), cannibalism (Newberry, 2004), and panic induced smothering (Mills and Faure, 1990; Boissy, 1995; Bright and Johnson, 2011; Richards et al., 2012; Barrett et al., 2014).
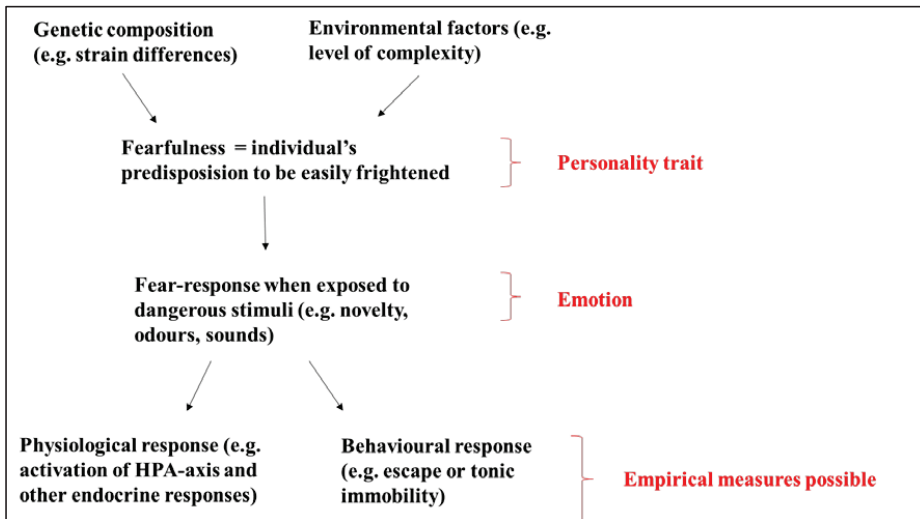
Figure 3. Schematic overview of the links between fearfulness, fear responses, and how measures of fear responses (behavioural and/or physiological) can be used to assess fear. Illustration: Margrethe Brantsæter.

1.4.1. Behavioural assessment of fear and fearfulness in poultry

As fear is crucial to the welfare of the individual, good methods to assess fear responses are required. The conscious component of fear cannot be directly measured (Watanabe, 2007; Mendl et al., 2010). The assessment of fear, therefore, relies on the assessment of behavioural and physiological responses when individuals are exposed to fear-inducing stimuli. To date, although physiological and neurobiological methods are increasingly common, most studies on affective states in laying hens are based on behavioural measures (Widowski et al., 2013). Unless stated otherwise, the fear responses included in this thesis were based on behavioural methods. Research on rodents suggests that animals forced into proximity with dangerous stimuli respond by immobility or aggressive behaviour (Blanchard et al., 1990). Fear responses in laying hens are either active avoidance (escape or hiding) or passive avoidance (tonic immobility) (Erhard and Mendl, 1999). Additionally, fear can inhibit normal activities (reduced movement, foraging, and social interaction), result in changed head and neck posture, be vocalised as alarm calls, and release particular pheromones (Boissy, 1995; Forkman et al., 2007). In situations that elicit an antipredator response, one option for the bird is to react with tonic immobility (Forkman et al., 2007). 'Death feigning' is a survival strategy that can cause the predator to lose interest in the prey (Sargeant and Eberhardt, 1975). Although the tonic immobility (TI) test is the most commonly used fear test in birds, it is of limited relevance when the aim is to assess the threshold at which the stimulus elicits a fear response (Forkman

et al., 2007). Conversely, it is better to assess the threshold that provokes a fear response in situations where the animal can move freely. A method commonly used to measure fear is to record the behavioural responses of approach and avoidance when birds are exposed to novelty (Forkman et al., 2007). The novel object test is less likely to be confounded by the handling required to conduct a TI test and may therefore be a better measure of fear than the TI test. Novel object tests are based on the assessment of the conflicting motivations to approach and avoid potentially dangerous stimuli. Miller described this in the approach-avoidance model (Miller, 1944; 1959) (Figure 4). As the animal approaches the stimuli, its motivation to approach as well as to avoid the stimuli increases. However, the motivation to avoid increases more sharply than the motivation to approach. Thus, according to Miller's model, an animal will approach the stimulus until the motivation to approach the stimulus is equal to the motivation to avoid it (Miller, 1944; 1959). An individual with a higher level of fearfulness will, therefore, keep a greater distance from the stimulus (have a higher motivation to avoid than to approach) when given a choice, compared to an animal with lower a level of fearfulness. Miller's model is the foundation for my interpretation of the fear responses in this thesis.
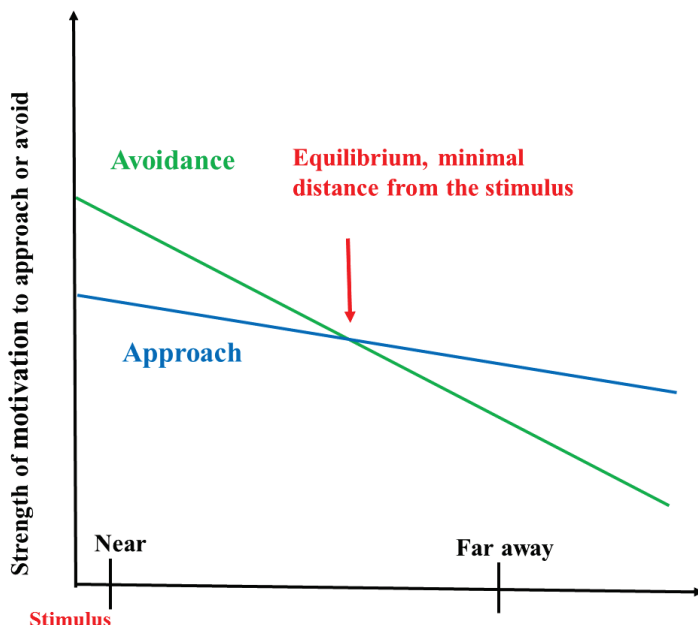
Figure 4. Miller's model of approach-avoidance. The animal will approach the stimulus until the point where the motivation to avoid and to approach are of equal strength (visualised by where the line for approach intersects the line for avoidance). Fearfulness will inhibit a fearful animal from approaching and thus the animal will keep a greater distance from the stimulus compared to a less fearful individual. Modified from Miller (1944).

Fear responses can be observed in the home environment or a separate test arena. Birds can be tested one by one or in groups. The observations can occur indirectly, using video recording, or by inspection. When the birds are observed in their familiar home environment, particularly using video recordings, the results are not confounded by disturbance due to human presence or handling. However, results based on controlled, experimental settings are not necessarily transferable to commercial farming conditions (Dawkins, 2012; Gilani et al., 2012).

Although welfare is a state experienced by the individual, flock-level measures are needed for welfare assessment in food-producing animals. In 2009, the Welfare Quality® (WQ) project published the Welfare Quality Assessment Protocol for Poultry (Welfare Quality, 2009). The WQ protocol addresses 12 welfare criteria divided into four principles: good feeding, good housing, good health, and appropriate behaviour. The protocol thus includes measures of all three approaches to animal welfare science (Figure 2). In previous welfare assessment protocols, environment- and management-based measures such as animal welfare indices (Bartussek, 2001) or ethical accounts (Sørensen et al., 2001) predominated. On the other hand, the WQ protocol highlights the importance including animal-based measures. Criteria number 12 in the WQ protocol states, 'Negative emotions such as fear, distress, frustration or apathy should be avoided whereas positive emotions such as security or contentment should be promoted' (Welfare Quality, 2009). The work presented in this thesis was limited to focus on fear and does not include measures of positive emotional states. An expert panel identified observation of feather appearance as the highest-ranking animal-based measure for laying hens (Whaytt et al., 2003). In the same study, observation of fear behaviours was ranked as the fifth most important animal-based measure in laying hens.

1.4.2. Physiological assessment of stress

Fear-inducing stimuli are potent stressors associated with activation of the hypothalamic–pituitary–adrenocortical (HPA) axis (Jones, 1987). Increased heart rate, increased core temperature, and drop in skin temperature are measurable physiological changes when hens are exposed to acute stressors (Cabanac and Aizawa, 2000). Short-term stress results in release of catecholamines (primarily adrenaline) and altered behaviour (e.g. flight) (Barnett and Hemsworth, 1990). If the response to the acute stressor is insufficient or the stressor persists, increased concentrations of biologically active substances (e.g. corticosterone) will aid

adaptation by mobilising the animal's reserves in an attempt to regain homoeostasis. In the wild, these mechanisms increase the chance of survival. However, chronically elevated levels of corticosterone can negatively influence health (Barnett and Hemsworth, 1990; El-Lethey et al., 2001; Forkman et al., 2007). In other words, chronic stress can lead to reduced welfare ('if stress increases, welfare decreases'; (Barnett and Hemsworth, 1990)).

In chickens, urine and faeces are eliminated together as droppings (Lepschy et al., 2008). However, the terminology used varies between different studies, so in this thesis 'defaecation' is considered to consist of both urinary and faecal components. Defaecation frequency is an indirect measure of stress (Espinosa-Medina et al., 2016). Several studies suggest that, although not as commonly reported in bird studies as in rodent studies (Hall, 1934; Boissy, 1995; Antoniadis and McDonald, 1999), this physiological assessment can also be useful in poultry research. Freezing and defaecation frequency were correlated when chicks were tested in a novel environment (Candland et al., 1963), thus indicating that both measures are affected by fear-inducing stimuli. Also, when exposed to novelty, cage-reared chicks with access to environmental enrichment had reduced defaecation frequency compared to cage-reared chicks without environmental enrichment (Candland et al., 1963). It was also been shown that individually tested chicks defaecated more often compared to chicks tested in pairs (Jones and Merry, 1988). Furthermore, birds of a low feather pecking line defaecated more in a novel environment compared to birds of a high feather pecking line (de Haas et al., 2010).

Possible methods of measuring HPA-axis activity include concentration of corticosterone and associated metabolites in plasma (Rettenbacher et al., 2004; Mostl et al., 2005; Rettenbacher and Palme, 2009) or in droppings (Palme, 2005; Palme et al., 2005; Palme, 2012; Palme et al., 2013). If the aim is to assess acute stress responses, analysis of plasma samples is ideal (Mormède et al., 2007). The concentration of faecal corticosterone metabolites (FCM) is an indirect measure of the level of circulating plasma corticosterone, so FCM represent the cumulative secretion of stress hormones over time (Palme, 2012; Palme et al., 2013). Thus, the levels detected in faecal samples are less affected by short fluctuations of hormone secretion compared those in plasma concentrations (Palme, 2012). Handling is a potent stressor in laying hens. Analysis of FCM is non-invasive and requires limited disturbance of the animals. Faecal analysis is the preferred method to assess hormones related to stress (Dawkins, 2004; Mostl et al., 2005; Palme et al., 2013). In laying hens prevented access to litter, increased FCM concentration correlated with decreased willingness to approach a novel object (Alm et al.,

2015). Similarly, laying hens prohibited access to nest boxes (Alm et al., 2016) or exposed to unpredictable feed restriction (Janczak et al., 2007a; Janczak et al., 2007b) over time showed increased concentrations of FCM. Thus, assessment of FCM seems suitable to assess effects of fear-inducing stimuli and stress in laying hens.

1.5. Problem behaviours in laying hens

In laying hens, fear is associated with behaviours such as feather pecking (Uitdehaag et al., 2009; de Haas et al., 2010; de Haas et al., 2013; Kops et al., 2013; Rodenburg et al., 2013), cannibalism (Newberry, 2004), and fear-induced smothering (Mills and Faure, 1990; Boissy, 1995; Bright and Johnson, 2011; Richards et al., 2012; Barrett et al., 2014). Moreover, commercial egg farmers report problems that are unlikely to be related to fear, such as toe pecking, social clumping, reduced plumage quality, and laying eggs outside the nest boxes. Problem behaviours are defined as behaviours that are problematic for the person reporting the behaviour (Mills, 2003). According to Mills (2003), three categories of problem behaviours have been classified: a) behaviours that have adaptive value for the given species, but that are inconvenient for the keeper; b) behaviours that are attempts to behave in an adaptive way in an environment that does not allow for complete adaptation; and c) behaviours that express disruption of the nervous system. Eggs laid outside the nest boxes increase the labour cost for the farmer and are, therefore, mainly a problem for the producer. The majority of the problems reported by egg producers, however, not only result in negative economic consequences but also compromise laying hen welfare (Waiblinger et al., 2006) (Table 1). In this thesis, the term 'problem behaviour' is not limited to Mills's definition (2003) but also includes behaviours that compromise laying hen welfare. In the following sections, the most important of these problem behaviours and their associations with fear are briefly discussed.

Table 1. Overview of problem behaviours and consequences for animal welfare and the farmer. Modified from Jones (1996).

Problem behaviour	Consequence
Panic/hysteria resulting in uncontrolled movements (smothering or crashing into walls, perches etc.)	Injuries (lesions or wounds e.g. broken wings, damaged/broken keel bones) or mortality (suffocation)
Feather pecking, feather damage, and feather loss	Skin lesions, pain, increased feed intake, and reduced feed conversion ratio
Inhibition of other motivational systems	Reduced adaptability, decreased use of available resources (e.g. perches, dustbathing area, or nest boxes)
Avoidance of stock people; animals are difficult to examine and/or handle	Management more time consuming/labour intensive → economic loss
Energy wastage (delayed maturation and/or reduced feed conversion ratio)	Reduced egg quality and/or egg mass, → Economic loss
Disturbed ovulation process	Egg shell abnormalities → economic loss

1.5.1. Panic and hysteria

In poultry, panic or violent escape reactions are consequences of inappropriate or exaggerated fear responses (Boissy, 1995; Richards et al., 2012). The behavioural priority to move away from the perceived threat may override all other considerations and result in flock panic or 'hysteria' (EFSA, 2005). Panic or hysteria can spread through the flock via social transmission (Mills, 2003). A fear response initiated by one or a few individuals can result in most or all of the birds in the flock reacting simultaneously. When birds panic or are hysterical, they often run into obstacles or pile on top of and trample or smother each other. Birds at the bottom of the heap may suffocate, and others may suffer broken bones (e.g. keel bone fractures (Harlander et al., 2015)), cuts, and scratches (Hansen, 1976; Mills and Faure, 1990), leading to increased mortality rates (Hansen, 1976; Mills and Faure, 1990; Jones, 1996; Hegelund et al., 2006; Waiblinger et al., 2006). The injuries cause pain (Nasr et al., 2012b; 2015) and wounds predispose the bird to infections, physical debilitation, and behavioural symptoms such as social withdrawal (Jones, 1996). In addition to the negative welfare consequences, these fear responses compromise production results and income for the farmer (Jones, 1996) (Table 1). Generations of domestication have reduced chickens' fear of humans (Campler et al., 2009), but predator-avoidance behaviours still occur in farmed species (Boissy, 1995; Forkman et al., 2007). In fact, exposure to humans (visual, auditory, or physical) (Jones, 1996; Waiblinger et

al., 2006; Edwards et al., 2013) is one of the most common causes of panic reactions in laying hens. Panic reactions are also related to sudden or novel changes to the physical or social environment. Examples of panic-inducing stimuli include exposure to unknown stock people, the farmer's wearing different coloured clothing, new smells, sudden and/or unfamiliar noises, transportation, and re-grouping of individuals (Boissy, 1995; Jones, 1996; Davis and Taylor, 2001). Situations that involved exposure to a combination of unfamiliar humans and unknown objects were associated with outbreaks of panic in free-range hens (Richards et al., 2012). In the cited study, more panic episodes occurred when the birds were young (25 weeks) compared to later in the laying period. This result could be due to the birds' habituating to the production environment over time (Richards et al., 2012). While panic usually can be linked to a disturbing event, 'hysterical' birds show the same behavioural responses as during panic, but with no observed external stimulus (Hansen, 1976). A group of hens are considered to be 'hysterical' if these episodes occur regularly (Laycock and Ball, 1990).

1.5.2. Inhibition of other behaviours

Fear is a powerful emotion that inhibits other motivated behaviours (Jones, 1987). The time spent foraging, exploring, or interacting with conspecifics (socially or sexually) decreases in fearful birds. Consequently, the birds' ability to cope with environmental changes, to utilise (new) resources, and to interact successfully with conspecifics is reduced (Jones, 1996). Fear might, therefore, inhibit the birds' ability to deal with the stressful experience involved in the transfer from the rearing to the production farm (e.g. handling, transportation, and interruption of social groups).

1.5.3. Pecking-related problem behaviours: feather pecking and cannibalism

Feather pecking is considered to be a multifactorial behaviour (reviewed by Hartcher et al., 2016). Severe feather pecking, when the recipient is injured as a result of the pecking, is one of the main welfare issues in modern egg production worldwide (Nicol et al., 2013). Of relevance to this thesis is the link between fear and severe feather pecking, a link that is not clearly understood (reviewed by Van Krimpen et al., 2005). Pullets that displayed higher fear responses and were less social in open-field tests showed higher levels of feather pecking as adults (Jones et al., 1995; Rodenburg et al., 2004). De Haas et al. (2014a, b) found that fear of humans during rearing was predictive of a higher occurrence of feather pecking and reduced plumage quality, both during rearing and during lay (de Haas et al., 2014a; de Haas et al.,

2014b). Overall, these studies suggest that having higher levels of fearfulness during rearing is a risk factor for developing feather pecking during lay. Also, others have found that birds that are more fearful when young have a higher risk of becoming victims of severe feather pecking (Uitdehaag et al., 2009). On the contrary, other studies indicate that increased fearfulness is a consequence of feather pecking, induced by tissue damage and pain (Blokhuis and Haar, 1992; Vestergaard et al., 1993; Hansen and Braastad, 1994; Bolhuis et al., 2009), rather than the other way around. Overall, these studies emphasise that fearfulness and severe feather pecking are interlinked and that reducing one might positively influence the other.

Cannibalism is defined as consuming tissues of other members of the same species (Newberry, 2004). Severe feather pecking and cannibalistic pecking of feathered areas are positively correlated (Cloutier et al., 2000). Thus, factors that prevent or stimulate feather pecking affect cannibalism simultaneously (Knierim, 2006). Accordingly, one can assume that the relationship between cannibalism and fear is as complicated as that of severe feather pecking (see previous paragraph). Cannibalism is most commonly reported during lay but has also been demonstrated during rearing (Johnsen et al., 1998; Riber et al., 2007). Cannibalism often occurs as 'outbreaks', which could be due to social learning (Tablante et al., 2000; Cloutier et al., 2002; Newberry, 2004). Cannibalism is reported from both cages (Tablante et al., 2000) and loose-housed systems (Newberry, 2004). However, due to the higher number of potential victims, cannibalism is considered a more severe welfare issue in loose-housed birds. As most birds in Norway are loose-housed, and beak trimming is banned, cannibalism is a relevant welfare issue of concern.

1.6. Previous work on rearing effects on fear in laying hens

Previous work on laying hens has detected several factors that may affect fear responses. The literature suggests that factors such as genetics (Schütz et al., 2001; Oden et al., 2002; Jensen, 2006; Uitdehaag et al., 2008; de Haas et al., 2014b) and a range of environmental factors (Boissy, 1995; Jones, 1996; Ohl et al., 2008) are relevant. A complete overview of all the potential factors known to affect fear responses is outside the scope of this thesis. The work presented in this thesis focused on effects of environmental complexity during rearing on fear responses in adult laying hens after transfer to the production facility.

The housing system used during rearing is the most important factor determining the level of environmental complexity to which the birds are exposed. The literature suggests that increased environmental complexity reduces fear responses in laying hens compared to rearing in barren environments (Johnsen et al., 1998; Colson et al., 2006). The study by Johnsen et al. (1998) compared floor rearing (rather than aviaries) with cages. Due to the current legislation, the cages used in the mentioned studies are no longer permitted in Norway, and floor rearing is no longer common in commercial rearing of laying hens. The other study compared rearing in floor pens and rearing in aviaries (Colson et al., 2006). However, the combinations of rearing housing and production housing were not standardised across the experimental groups, so the effect of rearing housing cannot be disentangled from the effect of the housing at the production farm.

The other principal factor that determines the birds' exposure to environmental complexity is whether they have access to environmental enrichment or not. Previous studies suggest that access to environmental enrichment reduces fear in laying hens (Candland et al., 1963; Jones and Waddington, 1992; Reed et al., 1993; Brake et al., 1994). However, most research on the effects of environmental enrichment has been conducted using cage-reared (Candland et al., 1963; Jones and Waddington, 1992) or floor-reared (Reed et al., 1993), rather than aviary-reared, birds. In the majority of the cited studies, the fear tests were conducted while the birds were in the rearing environment. Therefore, the rearing effect might be confounded with effects of the environment at the time of the study (Candland et al., 1963; Jones and Waddington, 1992; Brake et al., 1994). Other studies have used fear tests that deviate greatly from validated fear tests (Reed et al., 1993) or fail to provide information about factors that also may affect fear responses (e.g. stocking density, group sizes, beak trimming, and light strength (Candland et al., 1963; Brake et al., 1994; Colson et al., 2006). Without this information, it is difficult to interpret the results.

In experimental studies, the housing (design of pen, group size, and stocking density) and management often differ remarkably from conditions during commercial rearing and egg production (Jones and Waddington, 1992; Brake et al., 1994; Johnsen et al., 1998). The results obtained in experiments are therefore not necessarily transferable to commercial farms. Furthermore, despite indications that provision of inanimate objects reduces fear responses under controlled experimental conditions, commercial rearing farmers are unlikely to implement these labour-intensive strategies in their management. A procedure that can increase

environmental complexity under commercial rearing is the provision of a pecking substrate (litter) to pullets before they are released from aviary rows. Chicks that experienced disruption or limitation of litter supply kept a larger distance to a human and had increased latency to approach a novel object compared to birds reared with constant access to litter during early rearing (de Haas et al., 2014b). However, in the cited study all chicks were originally given access to litter, so the effect of no access to a pecking substrate was not investigated.

1.7. Knowledge gaps

There is limited knowledge on how the transfer from a loose-housed aviary to a furnished cage affects fear responses in adult laying hens. Previous studies in this field are not designed appropriately to avoid confounding the rearing treatments with other potential confounders. The majority of pullets in Norway are reared in aviary systems, and most of the relevant studies compare floor rearing rather than aviary-rearing loose-housed systems, so these studies might be inadequate. Furthermore, little knowledge exists on how rearing in a complex aviary system affects fear responses compared to rearing in barren cage environments. This was the first knowledge gap addressed by this thesis.

Commercially applicable methods to reduce fear responses in aviary rearing systems are currently lacking. The third study addressed whether provision of a pecking substrate during early rearing reduced fear responses observed in adult laying hens.

Finally, egg producers sometimes complain about feather pecking, cannibalism, hysterical hens, feather loss, and other problem behaviours in adult birds. However, to date, there is little information about risk factors during rearing and production associated with these issues.

2. Aims and objectives

The overall aim of this thesis is to increase knowledge on how aspects of the rearing period influence fear responses in adult laying hens. The main aim was approached through studies addressing the identified knowledge gaps. The aims and specific objectives were as follows:

Objective 1: Effects of early environmental complexity

The aim was to compare the effects of aviary rearing and cage rearing on fear responses in adult laying hens (papers **I** and **II**). Also, the effect of environmental complexity on spatial distribution in the home pen and indicators of basal levels of stress were measured (paper **II**).

Objective 2: Effects of early provision of a pecking substrate

This part of the project aimed to test whether the provision of a pecking substrate from the first day of life reduced fear responses in adult laying hens under commercial egg farming conditions (paper **III**).

Objective 3: Risk factors associated with problem behaviours in adult laying hens

The aim of this study was to identify rearing-related and production-related risk factors associated with the observation of problem behaviours in laying hen flocks, based on self-reported information from egg producers (paper **IV**).

3. Materials and Methods

3.1. Experimental animals

In the studies in this thesis, both the main breeds used for commercial egg production in Norway, namely Lohmann Selected Leghorns and Dekalb White, were represented. All birds in the studies were non-beak-trimmed laying hens of normal health status. In papers **I** (Lohman LSL), **II** (Dekalb White), and **III** (Lohmann LSL), only white laying hens were included, whereas paper **IV** also included ISA Brown and Lohmann Brown. The age of the birds in the different papers varied from 0–21 weeks of age (paper **I**), 0–23 weeks of age (paper **II**), 0–32 weeks of age (paper **III**) and 0–78 weeks of age (paper **IV**). The use of experimental animals was approved by the Institutional Animal Care and Use Committee at the Norwegian University of Life Sciences.

3.2. Housing conditions

3.2.1. Rearing conditions

All the animals included in this research were reared on commercial rearing farms. For the rearing farmers in paper **III**, the provision of paper inside the aviary rows was controlled by the experimental setup. All other husbandry procedures (light setting, feeding, inspections, etc.) were standard and based on the recommendations by the management guide provided by the breeding company. Incubation and hatching of the animals included in papers **I**, **II,** and **III** occurred at commercial hatcheries.

After hatching, the birds in paper **I** were reared either in a room containing an aviary or in a room with rearing cages. All other management procedures were identical. At 16 weeks of age, 3,750 aviary-reared hens and 3,750 cage-reared hens were transported to the same furnished cage production farm (n = 7,500).

The birds included in paper **II** were transported from the commercial hatchery to an aviary rearing farm. At five weeks of age, half of the birds were released into the aviary corridors (the standard procedure for aviary-reared birds) (Photo 7). However, the remaining birds were confined inside the aviary row for the entire rearing period, imitating cage rearing. All other

aspects of the rearing experienced by the birds were identical. At all times, the groups were separated by wire mesh to ensure that the farmer distributed flocks of either aviary-reared or cage-reared birds to the egg producers at 16 weeks of age.

Photo 7: Picture from the rearing farm in paper **II**. After five weeks of age, the aviary-reared birds were released into the corridor, as seen on the left side of the picture. On the right side of the photo, the birds confined inside the aviary row, imitating cage rearing, can be seen. Photo credit: Andreas Salte.

In paper **III**, 12 rearing farmers using aviaries were willing to participate in the study. However, only five of them had the appropriate house design and were enrolled in the study. The rearing farmers closed the divisions between the aviary rows of the system to stop the animals from moving between corridors. This study design enabled the birds to be reared under the same conditions, but in separate groups within the same house. In one of these groups, the rearing farmers covered the wire mesh floor inside the aviary rows with paper. Feed particles, dust, and faeces accumulated on the paper, forming a pecking substrate. No paper was supplied for the control group, situated in another row within the same house. Thus, the animals in the control rows were standing on bare wire mesh until the day they were let out onto the floor. At five or six weeks of age, depending on the rearing farmer's routines, the side doors to the aviary rows were opened. The animals were henceforth allowed to move freely within each corridor containing birds receiving the same treatment. At 16 weeks of age, the hens were transported

from the rearing farm to an aviary egg production farm. Each production farm received hens from either the paper-reared group or from the control group.

Originally, the plan for the study presented in paper **IV** was to compare questionnaire responses from the rearing farmers with those from the egg producers. However, we didn't manage to recruit enough rearing farmers, so the study ended up only including the questionnaires answered by the egg producers. To ensure a minimum of information about the rearing conditions experienced by the birds in paper **IV**, the egg producer questionnaire included a few questions about the rearing. The egg producer replies included information about the date of hatching, the name of the rearing farmer, the type of housing system the rearing farmer used, and the age of transfer from the rearing farm to the egg production farm.

3.2.2. Experimental housing

At 16 weeks of age, the 7,500 birds included in paper **I** – half of them reared in cages, and the other half reared in an aviary – were transported to the same furnished cage production farm. According to EU regulations, each cage unit can house up to 10 birds per cage. However, Norwegian regulations are stricter, so each furnished cage contained a maximum of nine birds. Each furnished cage housed either aviary-reared or cage-reared birds. The furnished cages were tiered in three levels (top, middle, and bottom) and arranged in four rows. Each row contained either aviary-reared or cage-reared birds. All other husbandry procedures were standard and followed the management guide provided by Lohmann Tierzucht (Lohmann, 2014).

At 16 weeks of age, the 120 aviary-reared and the 120 cage-reared birds in paper **II** were transported to the experimental facilities. Upon arrival at the experimental facilities, they were distributed in custom-built pens. Each pen contained 12 birds in mixed groups of six aviary-reared birds and six cage-reared birds. All husbandry procedures were standard and followed the recommendations from the Dekalb Management Guide (ISA Hendrix Genetics, 2014).

In paper **III**, each egg producer received a flock of birds either reared on paper (treatment) or reared on wire mesh (control) for the first five weeks of life. Most of the time, the egg producers were unaware that the hens were participating in a research project. This ensured that they treated their flocks as normal to avoid confounding of the rearing effect. The farmers who knew about the research were blinded as to which of the treatments the birds had been given.

Researchers visited the flocks at 30 weeks of age and assessed the birds in two fear tests: a novel object test and a stationary person test (SPT) (see section 3.3.4.).

Paper **IV** included birds in several different housing systems. Of the 78 flocks in the final data set, 40 (51%) were housed in aviary systems, 30 (38%) were in furnished cages, and 8 (11%) were housed in floor systems, aviaries with access to outdoor areas, or in organic aviary systems.

3.3. Behavioural assessment of fear

The methods used to assess fear presented in this thesis have been validated by others (Rushen, 2003; Waiblinger et al., 2006; Forkman et al., 2007). The fear tests were conducted in the home cage (paper **I**), in the aviary corridors (paper **III**), or in a separate test arena (paper **II**). Novel object tests were performed in papers **I**, **II** and **III**, whereas voluntary human approach tests were conducted in papers **II** and **III**. Additionally, the startle response (flight vs. no flight reaction) when exposed to an unexpected movement by a novel object was assessed in paper **II**.

3.3.1. Fear test in the home cage (paper I)

3.3.1.1. Data collection

Data collection in paper **I** consisted of video recordings of a selection of cage units when the birds were 19 and 21 weeks of age. The recordings consisted of two parts. First, the birds were filmed undisturbed for 10 min. Afterwards, a researcher returned and added a novel object to the cages. The researcher then left the room, and the birds were filmed for another 10-min session. The novel object used was an empty plastic bottle, hung with a wire attachment on the front bars of the cage. This left the bottle just inside the cage, approximately 10 cm from its right boundary (Figure 5).
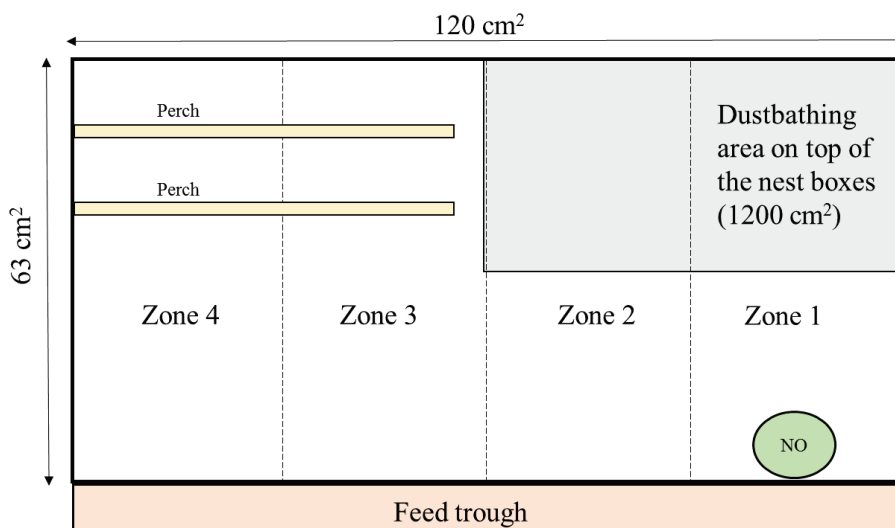
Figure 5. Rough schematic presentation of the furnished cages at the production farm (7560 cm²) that housed groups of maximum nine birds. During the novel object test, the novel object (NO), an empty plastic bottle, was positioned in zone 1, towards the front of the cage. During analysis, the cage was divided into four equally wide zones with increasing distance to the novel object. Illustration: Margrethe Brantsæter.

3.3.1.2. Data management

Observer XT 7.0 software was used for behavioural analysis of the footage. The behavioural analysis was based on the video recordings, made by a single researcher who was blind to the rearing background of the birds. Before beginning the observational analysis, a focal bird was chosen at random. Observations began 1 min after placement of the novel object and measured the duration of time the focal bird spent in the different zones after introducing the novel object. The zone with the novel object was coded as zone 1. The remaining zones had decreasing proximity to the novel object.

3.3.1.3. Statistical analysis

In paper **I**, the cage was the statistical unit. The ANOVA model that was used included the factors rearing treatment, tier (top, middle, or bottom), and the interaction between treatment and tier. All factors were fixed. The ANOVA results are presented as F-values and p-values. Means and SD are presented for the raw, untransformed data. The duration of time spent in the ¼, ½, and ¾ of the cage closest to or containing the novel object were used as indicating proximity to the novel object. If needed, these variables were Box–Cox transformed to meet

the assumptions of a GLM. Data for the two ages (19 and 21 weeks of age) were analysed separately. Results were considered statistically significant when p-values were ≤ 0.05, and as tendencies when 0.05 < p < 0.1. All statistical analysis was performed using JMP version 11.0 (SAS Institute Inc., NC, USA).

3.3.2. Fear tests in a test arena (paper II)

The behavioural fear tests in paper **II** were performed at 19 weeks (n = 80) and 23 weeks of age (n = 80). Each bird was tested at one age only. All the birds were assessed in a voluntary human approach test and a novel object (NO) test. The test periods lasted four days. During each test period, two birds from 10 different pens (five pens per room) were tested daily. Two aviary-reared birds and two cage-reared birds from each pen were tested.

The test room was next door to the rooms where the birds were housed. The test arena measured 210 cm by 180 cm by 120 cm (length × width × height). Three of the walls were black and opaque, whereas the fourth wall consisted of netting and was, therefore, transparent. The human or the NO was positioned 20 cm outside the netting. When sitting in front of the arena, the stimulus person in the human approach test looked directly toward the arena. The birds were recorded using two different cameras. One (Panasonic, WV-CP500/G) was suspended from the ceiling, positioned so that it faced the middle of the test arena. This camera was connected to a computer with EthoVision XT 10 (Noldus Information Technology, Wageningen, the Netherlands). The other camera was a camcorder (Canon, Legria HFM56) mounted on a tripod. This camera recorded birds from a position adjacent to the human or NO (Figure 6).
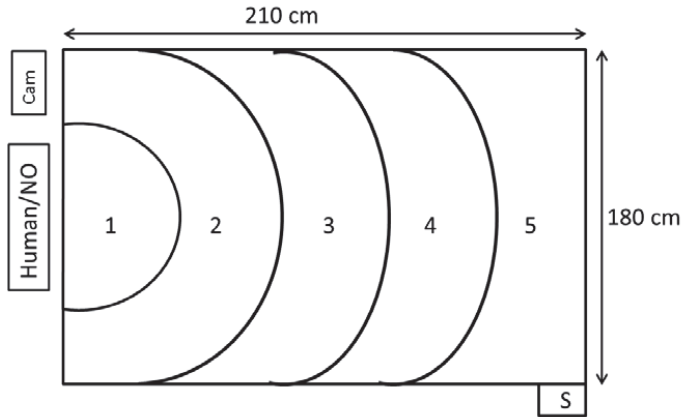
Figure 6. Schematic drawing of the test arena. The bird entered the arena from the start box (S) in zone 5, furthest away from the human or novel object (NO). The camera (cam) was positioned next to the human/novel object. The wall between zone 1 and the human/novel object was transparent netting to enable the birds to see the human/novel object. Illustration: Margrethe Brantsæter

3.3.2.1. Data collection

First, the bird was carried from the home pen and positioned in the start box. As soon as the stimulus human was placed on the chair, another researcher released the bird from the start box into zone 5. The duration of the voluntary human approach test was 5 min. After testing, the bird was left in the arena and immediately exposed to the NO test.

Immediately after the voluntary human approach test, the stimulus person had 5 s to open the beige umbrella, place it on the chair, and move out of sight. The flight response when the umbrella was opened at the beginning of the novel object test was scored. The duration of the NO test was 5 min. Directly after testing, birds were marked with a yellow ring tag around the left leg to ensure no birds were tested twice and returned to the home pen.

3.3.2.2. Data management

EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands) calculated the distance moved and the total time spent in areas of the arena closest to the stimuli (zones 1–4). The amount of time spent standing still and the birds' response when the umbrella was opened at the beginning of the NO test were categorised as flight or no flight by an observer blinded to the group to which each bird belonged.

The correlation between the continuous test variables in the behavioural tests was analysed using principal component analysis (PCA) to interpret and reduce the number of variables. The variables included in the PCA were the total distance moved, time spent standing still, and the amount of time spent close to the human or NO. The PCA, therefore, included a total of six variables. The component that was retained was used to generate component scores for individual birds to test for treatment effects using ANOVA. Principal component scores were checked to confirm that they fulfilled the assumptions of general linear models. The ANOVA model was Y = pen' + treatment + pen' × treatment (pen was a random factor and treatment was fixed). Because two hens from the same treatment were tested from each pen, we used pen as the experimental. PCA and ANOVA were conducted in JMP version 11.0 (SAS Institute Inc., NC, USA).

The effect of treatment on whether birds showed a flight response was analysed using logistic regression. The analysis was run separately for the two ages (19 and 23 weeks of age). Both treatment (aviary vs. cage) and the zone (1–5) in which the bird was positioned when the umbrella opened were included in the model. For treatment, aviary-reared birds were compared to cage-reared birds, and for zone, zones 1, 2, 3, and 4 were compared with zone 5. Odds ratios (OR) and p-values are reported.

The relationship between individual scores for the principal component related to fearfulness and the flight response, when exposed to the umbrella, was tested by logistic regression. Flight response was the dependent variable, and the principal component score was the independent covariate. Logistic regression analysis was conducted in Stata (STATA SE 14.0 for Windows).

Results were considered statistically significant when p-values were ≤ 0.05 and as tendencies when $0.05 < p < 0.1$.

3.3.3. Use of elevated areas in the home pens (paper II)

3.3.3.1. Data collection

The spatial distribution of birds in the home pen was recorded at 19 and 23 weeks of age. The observer walked down the aisle of the room, counting the birds from each rearing treatment

that were a) perching on the upper perch, b) placed on the elevated platform, c) perching on the low perch, or d) positioned on the floor.

3.3.3.2. Data management

The hens from each treatment positioned on the top perch, the elevated platform, and the low perch were counted. These numbers were then divided by the total number of hens from the relevant treatment to give the percentage of birds from each treatment found on the three different levels. We then calculated the average number of birds over the four days in each position in the pen during the periods of observation.

3.3.3.3. Statistical analysis

The resulting data were analysed by Wilcoxon matched-pairs signed-rank sum test. The relative numbers of aviary-reared birds and cage-reared birds in each position were treated as matched pairs. The results are presented as median, 25th and 75th percentiles for the different locations of the home pen. Results were considered statistically significant when p-values were ≤ 0.05 and as tendencies when $0.05 < p < 0.1$. The analysis was conducted in JMP version 11.0 (SAS Institute Inc., NC, USA).

3.3.4. Fear tests in commercial aviaries (paper III)

Data collection occurred when the birds were around 30 weeks of age, the peak of lay. Each farm contained on average 7,500 laying hens from the same rearing farmer and the same treatment group (paper or control). Both producers and researchers were blind to which treatment group the visited flock belonged. During this visit, the hens were subjected to two different fear tests: a stationary person test and a novel object test, as described below.

3.3.4.1. Data collection

The fear tests were each performed at six locations in the henhouse, the stationary person test always before the novel object test. The stationary person test was based on methods described by the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009), and papers evaluating fear tests in loose housing systems (Raubek et al., 2007; Graml et al., 2008a; Graml et al., 2008b; de Haas et al., 2014a; de Haas et al., 2014b). The experimenter walked slowly

down the corridor and stopped at six areas distributed throughout the house. At the given location, the experimenter would stand still for a total of 2 min. Every 10 s, aided by a stopwatch, the experimenter counted the number of birds within 25 cm of his or her feet. After the first three farm visits, the protocol was amended to include the number of birds within 2 m of the experimenter's feet. At the same location, after completion of the stationary person test, the experimenter proceeded with the novel object test.

The novel object test was based on the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009) and previous protocols conducted on loose-housed hens (de Haas et al., 2014a; de Haas et al., 2014b). The novel object used was a 50 cm long stick with different coloured tapes of 3 cm width. The novel object was placed on the floor of the corridor not too far from a light source, and the experimenter stepped slowly backwards approximately 1.5 m. After placement, every 10 s, the experimenter counted the number of hens within 25 cm (bird length) of the novel object. The test lasted a total of 2 min; the maximum latency to approach the novel object was thus 120 s.

In total, five rearing farms were included in the study. They generated 23 flocks of laying hens that met the inclusion criteria: 11 flocks reared without access to a pecking substrate (control) and 12 flocks reared with access to a pecking substrate (treatment group) during the first weeks of life. Measures from the novel object test and the stationary person test 25 cm were obtained at all 23 farms, whereas the stationary person test 2 m measure was collected at 20 farms only.

3.3.4.2. Data analysis

Measures from the SPT and the NOT at the six locations were averaged per flock. The response variables generated from the fear tests and the statistical method and model used are presented in Table 2.

3.3.4.3. Statistical analysis

Models for analysis of variance (ANOVA) were decided by backward and forward stepwise selection. Models with the highest R^2-adjusted values were selected for data analysis. All factors included in the final models had p-values < 0.1. The variables recorded during the visits that were possible to include in the models were the treatment group (access to paper versus

control), the rearing farm, whether the birds had access to environmental enrichment at the production farm and the age of the flock at the time of the visit. The statistical models used are shown in Table 2. All explanatory variables were fixed. The final model included the fixed factors treatment and enrichment. The assumptions of ANOVA were checked, and the variables were transformed if necessary. Post hoc testing was performed with Tukey's test (Tukey's HSD test). Presented values are untransformed means ± standard deviations for results analysed by ANOVA. Whether the birds approached within 25 cm of the stationary person was analysed by logistic regression. Results were considered statistically significant when p-values were ≤ 0.05 and as tendencies when $0.05 < p < 0.1$. The statistical software used was Jmp version 11 (SAS Institute Inc., NC, USA) for ANOVA and Stata SE 14 (StataCorp LP) for logistic regression.

Table 2. Overview of the statistical models used in paper **III**. The novel object test (NOT) and the stationary person test (SPT) included measures of approach (yes/no), latency to approach, and number of birds per flock that approached.

Response variables	Description	Statistical method
NOT approach	Whether one or more hens came within 25 cm of the novel object (yes/no).	Descriptive
NOT latency	The latency for three hens to come within 25 cm of the novel object. Maximum latency 120 s.	y = treatment + rearing farm + enrichment + (treatment × rearing farm)
NOT average	The average number of birds in the flock that approached within 25 cm of the novel object.	y = treatment + enrichment + (treatment × enrichment)
SPT 2 m approach	Whether one or more birds came within 2 m of the stationary person (yes/no).	Descriptive
SPT 2 m latency	The latency for one or more hens to approach within 2 m of the stationary person. Maximal latency 120 sec.	y = treatment + rearing farm + enrichment + (treatment x rearing farm)
SPT 2 m average	The average number of birds in the flock that approached within 2 m of the stationary person.	y = treatment + rearing farm + enrichment + (treatment × enrichment)
SPT 25cm approach	Did birds in the flock approach within 25 cm of the stationary person (yes/no)?	Logistic regression y = treatment + enrichment

3.4. Physiological assessment of stress (paper II)

Measures of basal stress levels were evaluated in paper **II**. The physiological assessments consisted of defaecation frequency and subsequent analysis of faecal corticosterone metabolites from the collected faecal samples.

3.4.1. Defaecation frequency and faecal corticosterone metabolites (paper II)

3.4.1.1. Data collection

The number of droppings during the 10 min in the test arena (defaecation frequency) was recorded, and all faeces was collected for analysis of corticosterone metabolites. The faecal samples obtained were stored in a freezer at −80°C pending analysis using an enzyme immunoassay (EIA).

3.4.1.2. Data analysis

Droppings were extracted with 60% methanol (0.5 g + 5 ml; (Palme et al., 2013)) and corticosterone metabolites were measured in an aliquot (after 1:10 dilution in assay buffer) of the supernatant. The measurement was performed with an EIA, which has been successfully validated for non-invasive evaluation of adrenocortical activity in chickens (for details of the assay, see (Rettenbacher et al., 2004)). The extraction was performed at NMBU while the EIA was carried out at the University of Veterinary Medicine, Vienna.

3.4.1.3. Statistical analysis

The defaecation frequency was analysed using Fisher's exact test. The results are presented as median, 25th, and 75th percentiles.

A total of 148 faecal samples were obtained for analysis of corticosterone metabolites. The corticosterone metabolite data fulfilled the assumptions of the general linear model and were analysed using the same ANOVA model described for the principal component scores (Y = pen' + treatment + pen' × treatment) where pen was a random factor and treatment was fixed. The results for the concentration of corticosterone metabolites are presented as means ± SDs.

Results were considered statistically significant when p-values were ≤ 0.05 and as tendencies when $0.05 < p < 0.1$. The defaecation frequency and the faecal corticosterone metabolite analyses were carried out in JMP version 11.0 (SAS Institute Inc., NC, USA).

3.5. Producer-reported problem behaviours (paper IV)

Paper **IV** was executed using an Internet-based questionnaire. The questionnaire was created and distributed using Questback™. During the design of the survey, it was tested on industry advisors and a selection of egg producers to ensure the quality and relevance of the questions.

3.5.1. Data collection

The questionnaire was distributed to all the egg producers whose e-mail addresses were acquired during the data collection period (August through November 2015).

Respondents were instructed to reply for their current flock if the animals were over 60 weeks of age. If their animals were younger than 60 weeks of age at the time of the survey, the producer was asked to reply for their previous flock. If the respondent had several flocks simultaneously, he or she was asked to respond for one flock only.

The questionnaire was divided into four different parts: 1) General info about the farm; 2) the flock's production results; 3) the environment, climate, and management routines; and 4) behaviour of the birds from arrival around onset of lay until the flock was euthanised at 70–80 weeks of age.

3.5.2. Data analysis

First, the data were manually checked in Microsoft Excel (2013) to verify whether the respondent had replied according to the instructions. Only replies where the respondent had replied for one flock (not several) and where the birds were minimum 60 weeks of age were included for further analysis. Open answer data had to be labelled and coded appropriately for statistical analysis.

The outcomes included in the questionnaire and the definitions given to the producers are listed in Table 3. The outcomes were coded as binomial variables (0 = behaviour not observed; 1 =

behaviour observed by respondent). For feather quality, good plumage was coded as = 0; reduced plumage quality ≥ 60 weeks of age = 1).

A continuous outcome index variable was generated. By creating the index, we avoided multiple testing due to the seven different dependent variables and the high number of possible predictors.

Table 3. An overview of outcomes included in the questionnaire and the information the producers were provided to answer the questions.

Behaviour	Description available to the producers when responding to the questionnaire
Gentle feather pecking	Gentle pecking at, or removal of, feathers from own coat or from a conspecific
Severe feather pecking	More intense pecking at conspecific that causes skin lesions
Toe pecking	Pecking at their own toes with resulting skin lesions
Cannibalism	Pecking at a conspecific resulting in large skin lesions, profuse bleeding, dysfunctional/removed body parts or death
Social clumping	Some, or all, hens pile up for no apparent reason. The birds do not seem disturbed or frightened.
Hysteria / panic	Some, or all, hens abruptly, and for no obvious reason, run or fly to one end of the room. The animals appear frightened.
Floor eggs	Eggs laid outside the designated nest boxes
Feather quality around the time of euthanasia	1) The hens were fully covered with good quality feathers 2) The hens had small patches without feathers 3) The hens were more or less naked

3.5.3. Statistical analysis

In the first step of model building, all explanatory variables were screened individually to assess their association with the index variable. The screening was conducted using multilevel mixed-effects linear regression, with rearing farmer as a random effect. Only predictors with $p < 0.2$ were kept for step two, which was backward stepwise selection. Log likelihood tests were used to assess the overall significance of categorical variables with more than two levels.

The final model, which also included rearing farmer as a random effect, was selected based on backward stepwise selection. Only factors with p < 0.1 were retained in the final model.

Based on the results from the whole dataset, a decision was made to analyse subsets for each housing category, namely aviary systems and furnished cages. The models for these subsets were constructed according to the same procedure described for the dataset as a whole.

Results from the models are presented as β coefficient ± standard error. Results were considered statistically significant when p-values were ≤ 0.05 and as tendencies when 0.05 > p < 0.01. All statistical analyses were conducted using Stata SE 14 (StataCorp LP).

4. Summary of results

4.1. Paper I

This study tested whether rearing in aviaries, as opposed to cages, reduced the fear response of laying hens after transfer to furnished cages. Fear was evaluated by introducing a novel object in the furnished cage and observing the birds' approach-avoidance behaviour. At 19 and 21 weeks, the cage-reared birds spent more time away from the novel object compared to the aviary-reared birds. These results suggest that rearing in an aviary environment reduces the fear response towards a novel object up to the fifth week after transfer to a new housing system compared to rearing in cages.

4.2. Paper II

The primary aim of paper **II** was to test the hypothesis that laying hens reared in a complex aviary system would be less fearful and less sensitive to stress, and would use elevated areas of the pen more often as adults than hens reared in a cage environment. Fear tests were conducted in a separate arena at 19 and 23 weeks of age. Data were analysed using analysis of variance on individual scores for a fearfulness-related principal component. The results indicate that aviary-reared birds have lower levels of fearfulness compared with cage-reared birds at both 19 and 23 weeks of age. The groups did not differ in defaecation frequency or in the concentration of faecal corticosterone metabolites at either age. At 19 weeks, but not at 23 weeks of age, more aviary-reared birds spent time on the elevated areas in the home pens compared to the cage-reared birds. The results of this study support the hypothesis that increased environmental complexity during rearing reduces fearfulness of adult laying hens as assessed at 19 and 23 weeks of age.

4.3. Paper III

The aim of this study was to test the hypothesis that chicks with access to a pecking substrate during the first five weeks of life would display reduced fear responses as adult hens compared to birds reared without a pecking substrate. At 30 weeks of age, the birds were tested in a

stationary person test and a novel object test. All flocks, regardless of rearing treatment, had birds within 2 m of the stationary person and within 25 cm of the novel object. The number of birds approaching the novel object was affected by the interaction between access to a pecking substrate during rearing and provision of environmental enrichment as adults (Figure 7). Thus, the reduced fear response towards the novel object was only detected in chicks reared with a pecking substrate if the birds did not have access to environmental enrichment as adults. The results indicate that provision of a pecking substrate from the first day of life and access to environmental enrichment as adults affect fear responses to a novel object in adult laying hens.
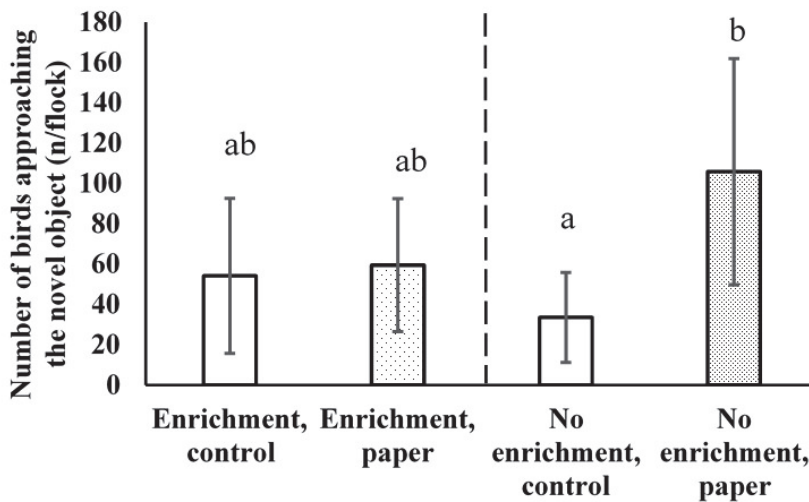
Figure 7. The bars display flocks from the control and paper rearing groups, and whether the birds were provided environmental enrichment as adults. Bars with different letters are significantly different ($p \leq 0.05$). Flocks reared with a pecking substrate, but without enrichment at the production farm, approached the novel object more than flocks reared without a pecking substrate and without enrichment as adults. Intermediate numbers of birds approached the novel object, irrespective of access to a pecking substrate during rearing, if they were provided enrichment at the production farm.

4.4. Paper IV

The aim of this study was to identify rearing and production-related risk factors associated with producer-reported problem behaviours in Norwegian laying hen flocks. Questionnaires were distributed to 410 egg producers, and 120 producers responded to the survey (response rate 29%). After exclusion of data that did not comply with the instructions, the final dataset included 78 flocks (19%). The main predictors associated with reported problem behaviours were housing system and issues with climatic conditions at the egg production farm. Egg

producers with aviary flocks reported on average 1.6 more problem behaviours compared to producers with furnished cages. Within aviaries (n = 40), the producers reported on average 1.7 more problem behaviours in flocks that experienced problems with climatic conditions compared to flocks without issues climatic problems. For respondents with furnished cages (n = 30), on average 1.1 fewer problem behaviours were reported in farms with at least 7,500 birds compared to farms with less than 7,500 birds.

5. Discussion

The studies presented in this thesis suggest that rearing in more complex environments, compared to rearing in barren environments, reduces fear responses in adult laying hens (papers **I**, **II** and **III**). Furthermore, papers **III** and **IV** emphasise that not only aspects of the rearing period but also housing system and management procedures at the production farm affect adult fear responses and associated problem behaviours in laying hens.

5.1. Effects of early life environmental complexity (papers I and II)

5.1.1. Fear responses (papers I and II)

In papers **I** and **II**, aviary-reared birds displayed reduced fear responses compared to cage-reared birds at 19, 21, and 23 weeks of age. In paper **I**, the aviary-reared birds spent more time close to the novel object compared to the cage-reared birds. In paper **II**, the aviary-reared birds were more active and spent more time adjacent to the stationary human and to a novel object compared to the cage-reared birds. The effect of environmental complexity during rearing was detected irrespective of whether the fear tests occurred in the home environment in the presence of cage-mates (paper **I**) or whether the birds were tested while socially isolated in a test arena (paper **II**). To the best of my knowledge, these are the first two studies documenting differences in fear responses of adult laying hens comparing aviary rearing rather than floor rearing with cage rearing in a study design that ensured control of confounding effects of the adult environment.

Birds reared in aviary systems are exposed to a higher level of environmental complexity compared to cage-reared birds (see sections 1.2.2. and 1.2.3). Also, the aviary-reared birds experience more potentially fear-inducing stimuli due to a wider range of novel situations and more interaction with humans. The aviary environment allows expression of wide range of innate behaviours and a greater possibility to escape aversive situations (e.g. bullying by other chicks or the farmer entering the room with unfamiliar tools) compared to birds housed in confined systems. In cage systems, more of the management is automated compared to the aviary-systems. Consequently, the time the rearing farmer spends in a henhouse with cages is

reduced compared to the time spent by a farmer with a rearing aviary. Furthermore, cage-reared birds are less exposed to human contact and episodes involving novel humans or objects. Overall, the fear tests in papers **I** and **II** indicate that the complexity of their environment makes the aviary-reared birds better prepared to deal with fear-inducing stimuli compared to the cage-reared birds. However, from our studies, it is not possible to conclude which aspects of the aviary-rearing are most important in reducing the fear responses. It could be that the environmental complexity, the exposure to a wider range of novel situations, increased human contact, or a combination of factors all contributes to these effects. To assess this further, a study design that controls the number of novel situations the birds are exposed to and standardises the interaction with the stock people would be necessary.

Animal welfare is conceptualised as a continuous spectrum ranging from 'very poor' to 'very good' (Fraser et al., 1997; Fraser, 2008; Hemsworth et al., 2015). For an animal's welfare to be at the positive end of the spectrum, it is not enough to reduce its fearfulness. Additionally, the environmental conditions and the management should allow the animal to experience positive feelings (i.e. experience positive affective states). As opposed to fear, which is considered an indicator of a negative affective state, alert behaviour is considered an indicator of positive welfare (Nicol et al., 2011). Birds from the same rearing treatments as the birds included in paper **I** were included in a parallel study (Tahamtani et al., 2014). At 19 weeks of age, the aviary-reared birds performed more alert behaviour and spent more time near the novel object at 19 weeks of age compared to the cage-reared birds (Tahamtani et al., 2014). Furthermore, the aviary-reared birds spent more time close to the novel object (paper **I**). These results could indicate that the welfare of the aviary-reared birds is towards the positive end of the spectrum. On the contrary, the cage-reared birds spent less time performing alert behaviour compared to the aviary-reared birds at both 19 and 21 weeks of age (Tahamtani et al., 2014). Additionally, the cage-reared birds spent less time in close proximity to the novel object (paper **I**). The behaviour of the cage-reared birds in both the results from Tahamtani et al. (2014) and paper **I** indicates that the cage-reared birds' welfare was further away from the positive end of the welfare spectrum. Fear is a potent inhibitor of other behaviours, so the cage-reared birds' reduced expression of alert behaviour could be linked to the results from the novel object test.

Interpreting the affective state of the aviary-reared birds at 21 weeks of age is more complicated. At this age, the aviary-reared birds still spent more time close to the novel object, but the time spent performing alert behaviour had decreased. A possible interpretation is that

transfer from a rearing aviary to furnished cages compromises the affective state of the aviary-reared birds with increasing age. This could add support to previous research suggesting that fear increases over time in cage-housed adult birds (Brake et al., 1994). Interestingly, the aviary-reared birds had a higher mortality rate throughout the production phase compared to the cage-reared birds (Tahamtani et al., 2014). The reason for the difference in mortality rates between the rearing groups is unknown. However, as affective state and biological function are interlinked, the decline in affective state could be among the influential factors for the increased mortality among the aviary-reared birds. Indeed, there were suggestions by Tahamtani et al. (2014) that the mortality among the aviary-reared birds was caused by frustration-induced aggressive pecking.

5.1.2. Use of elevated areas in the home pen (paper II)

In paper **II**, the use of elevated areas in the home pen was evaluated when the birds were 19 and 23 weeks of age. During rearing, the cage-reared birds could perch on metal bars inside the aviary row (see photos 4–6, section 1.2.2.). The aviary-reared birds had additional access to perches at different heights after release into the aviary corridors. Early experience with a more complex environment increases the birds' ability to use elevated perches and improves their ability to solve spatial tasks as adults (Gunnarsson et al., 1999; Gunnarsson et al., 2000; Kozak et al., 2016; Tahamtani et al., 2016b; Habinski et al., 2017). However, as fear is a potent behavioural inhibitor, fearful birds may be less active and have reduced motivation to explore the environment, and consequently be less inclined to utilise available resources. Indeed, the aviary-reared birds were observed significantly more often on the top perch, elevated platform, and low perch, compared to the cage-reared birds (who were mainly positioned on the floor) at 19 weeks of age. These results could indicate that the aviary-reared birds were more aware of perches and elevated areas of the pen or that they had developed better motor systems. The aviary-reared birds' superior use of the elevated areas could also be explained by their lower level of fear, as assessed in the behavioural fear tests, compared to the cage-reared birds. Similarly, the cage-reared birds' higher levels of fearfulness possibly prevented exploration and utilisation of the available resources. Alternatively, explanations for the cage-reared birds' poor use of the elevated areas at 19 weeks of age could include cognitive or physical deficits (Gunnarsson et al., 1999; Gunnarsson et al., 2000; Tahamtani et al., 2016b). The pens at the research facility were more complex than the cage-reared birds were used to, but more restrictive than the environment previously experienced by the aviary-reared birds. At 23 weeks

of age, there was no longer an effect of environmental complexity during rearing on the birds' use of elevated areas in the home pen. This result could indicate that the aviary-reared birds became gradually more similar to the cage-reared birds, or that the cage-reared birds learned to access the elevated areas with time. At this point, the majority of birds, irrespective of rearing group, were positioned on the floor of the pens.

5.1.3. Basal levels of stress (paper II)

In paper **II**, registrations of defaecation frequency and analysis of faecal corticosterone metabolites (FCM) assessed basal levels of stress. As the aviary-reared and cage-reared birds were mix-housed, samples were collected from the test arena after the birds had been tested.

Defaecation frequency is an indirect measure of stress (Espinosa-Medina et al., 2016). Several studies have detected increased defaecation frequency when birds are exposed to stressors (Candland et al., 1963; Jones and Merry, 1988; de Haas et al., 2010). Contrary to our predictions, the aviary-reared and the cage-reared birds did not differ in defaecation frequency while in the test arena. Because rearing affected fearfulness, as indicated by the PCA analysis, the lack of effect on defecation frequency could indicate that this is not an optimal indicator of stress in laying hens.

Because collection of faecal samples is non-invasive, analysis of faecal samples is the preferred method when measuring hormones related to stress (Dawkins, 2004; Mostl et al., 2005; Palme, 2012; Palme et al., 2013) (see section 1.4.2). Faecal corticosterone concentrations are affected by exposure to fear-inducing and frustrating stimuli (Janczak et al., 2007a; 2007b; Alm et al., 2015; 2016). Other factors known to affect concentrations of FCM are age (Nicol et al., 2006; Alm et al., 2014; 2015), sex, diurnal and seasonal variation (Dawkins, 2004; Dawkins et al., 2004), early life experience (pre- and post-natal) (Touma et al., 2003; Palme et al., 2005; Touma and Palme, 2005), diet (Klasing, 2005; Alm et al., 2014), housing system (Sherwin et al., 2010), genotype, metabolism, temperature, mass of droppings produced, and type of assay used (Goymann, 2012). In paper **II**, all these factors except early life rearing environment were standardised across the treatment groups. However, we did not detect a difference when comparing the concentration of FCM from aviary-reared versus cage-reared birds. So, the basal level of stress seemed unaffected by the level of environmental complexity during rearing. However, as for the defaecation frequency, we cannot exclude the possibility that an undetected

short-term rearing effect on the basal stress levels was gone by the time the faecal samples were taken. Furthermore, after an increased concentration of corticosterone plasma, it takes approximately four hours before this can be detected in the faeces (Dehnhard et al., 2003). It is possible be that the rearing affected the birds' response to a potent stressor, but as we only focused on basal levels of FCM, this was not included in our study.

5.2. Effects of access to a pecking substrate during rearing and environmental enrichment at the production farm (papers III and IV)

In paper **III**, more birds reared with early access to a pecking substrate approached the novel object compared to birds reared without a pecking substrate. However, this effect was only found in birds that were not provided environmental enrichment at the egg production farm. The results from paper **III** thus add to existing literature emphasising that behaviour is affected not only by the rearing period but also by the environment during adulthood (Jones, 1982; Reed et al., 1993; Nicol et al., 2001). Following the results reported by de Haas et al. (2014b), we had expected that birds reared with a pecking substrate but without access to enrichment as adults would be more fearful than birds with continuous access to a pecking substrate. The reason for the discrepancy between the results by de Haas et al. (2014b) and paper **III** is unknown. However, a possible explanation could be the difference in age of testing. De Haas et al. (2014b) tested the birds while the pullets were at the rearing farm (i.e. representing short-term effects), while the birds in paper **III** were tested as adults (i.e. representing long-term effects). In other words, if we had included additional tests of the pullets while they were still at the rearing farm, this could have provided us with more directly comparable results. Alternatively, the results by de Haas et al. (2014b) could be confounded with concurrent effects of pain after beak trimming due to long-term behavioural alterations (Gentle et al., 1990; Gentle et al., 1991; Davis et al., 2004; Janczak and Riber, 2015; Marino, 2017). For birds with access to environmental enrichment as adults, the access to a pecking substrate during rearing did not affect the result in the novel object test (paper **III**). One interpretation of this result is that enrichment given to adult hens counteracts negative effects of not having access to a pecking substrate early in life. In other words, if the rearing farmer does not provide the chicks with access to a pecking substrate, the egg producer might reduce the birds' fear responses by providing the birds with environmental enrichment later in life. With the impending European ban on beak trimming, the results from paper **III** encourage provision of a pecking substrate

during early life and environmental enrichment at the production farm as potential methods to reduce problems caused by fear responses in laying hens.

Although the literature supports the view that fear and feather pecking are interlinked, the causal relationship between the two is not clearly understood (reviewed by Van Krimpen et al., 2005; see section 1.5.3). In a parallel study of birds reared using the same treatments used for the birds in paper **III**, birds reared without access to a pecking substrate during rearing had higher odds of poor feather quality and higher incidence of gentle feather pecking compared to the paper-reared birds (Tahamtani et al., 2016a). The proximate mechanism for the reduced expression of fear in the paper-reared birds is not clarified by paper **III**. However, early provision of a pecking substrate results in concurrent improvement of feather quality, reduction of gentle feather pecking and reduced fear responses. These results could suggest that birds reared without a pecking substrate are more likely to develop feather pecking behaviour, which subsequently increases fear responses (Blokhuis and Haar, 1992; Vestergaard et al., 1993; Hansen and Braastad, 1994; Bolhuis et al., 2009). Alternatively, the increased fear in birds reared without a pecking substrate may have increased the chances of the birds developing feather pecking behaviour (Jones et al., 1995; Rodenburg et al., 2004; de Haas et al., 2014a; de Haas et al., 2014b) or becoming victims of feather pecking (Uitdehaag et al., 2009). Although the results from paper **III** do not answer the question of causation between fear and feather pecking, paper **III** supports the conclusion by Tahamtani et al. (2016a) that access to a pecking substrate during rearing is an applicable method to improve laying hen welfare under commercial conditions.

Contrary to the findings in paper **III**, provision of environmental enrichment was not associated with a decreased risk of problem behaviours (paper **IV**). This result was unexpected as several studies report that environmental enrichment prevents development of several problem behaviours (Nicol et al., 2001; de Jong et al., 2013; Rodenburg et al., 2013). The discrepancy between the results from paper **III** and paper **IV** could be due to the different methods used. In paper **III**, the researchers observed and classified whether environmental enrichment was present, whereas paper **IV** relied on the producers' interpretation of the questions. The producers might have had different opinions of what was meant by 'enrichment' than the researchers would. For example, among the furnished cage farmers, only one producer (3%) replied that the birds were not provided with environmental enrichment. As only one furnished cage producer replied 'no' to provision of environmental enrichment, it is valid to ask if some

of the producers considered the addition of nest boxes, perches, and a litter area, as required by the legislation (Landbruks- og matdepartementet, 2001), as 'enrichment'. If so, producers may have over-estimated the use of enrichment. Because the majority of the egg producers answered that enrichment was provided, this factor could not be included in the statistical analysis. Furthermore, from the data in paper **IV**, we cannot determine whether the producers provided enrichment in response to problem behaviours or as a preventative measure. Based on the types of enrichment observed by the researchers (paper **III**) and reported by the egg producers (paper **IV**), it seems that most egg farmers use traditional types of enrichment such as shell sand, pecking blocks, and pieces of plastic. Few farmers mentioned the use of auditory enrichment such as playing the radio. Auditory enrichment is another practical way to enrich the environment, which is associated with reduced fear responses in laying hens (Jones, 2004; Davila et al., 2011; de Haas et al., 2014a).

For birds housed in furnished cages, the dustbathing area is a considerable improvement compared to the battery cages. In paper **IV**, 40% of the furnished cage producers admitted that they block the hens' access to the dustbathing area upon arrival. Additionally, only 37% of the producers replied that they supplied dustbathing material at least once per week. These results are similar to those found in a Swedish survey, where 33% of respondents gave dustbathing material at least weekly (Svenska ägg, 2015). One bird using the dustbathing area is enough for the dustbathing material to be dispersed, so if the producer does not add dustbathing material regularly, the majority of the birds will not have access to dustbathing substrate. Preventing animals from performing highly motivated behaviours, such as dustbathing, can result in frustration and contribute to the development of harmful behaviours such as feather pecking or hysteria (Olsson et al., 2002; Olsson and Keeling, 2005; Lay et al., 2011). The results from paper **IV** indicate that, although required by legislation, the dustbathing area and provision of dustbathing material do not always function optimally in the furnished cage systems used in Norwegian egg production farms.

Overall, the results in papers **III** and **IV** correspond with the literature suggesting that the behaviour of adult birds is affected not only by the rearing environment but also by the environment experienced during adulthood (Jones, 1982; Reed et al., 1993; Nicol et al., 2001). Paper **III** suggests that both access to a pecking substrate during rearing and provision of environmental enrichment during production are promising methods that can reduce fear in adult laying hens. Paper **IV** indicates that an increasing number of egg producers provide

environmental enrichment. Based on the findings in paper **III** and paper **IV**, continued research on efficient and practicable methods to enrich the environment of laying hens both during rearing and at the production farm is encouraged.

5.3. Risk factors associated with problem behaviours (paper IV)

In paper **IV**, feather pecking, toe pecking, cannibalism, social clumping, smothering, floor eggs, and reduced feather quality were reported by 18%–60% of the egg producers. Fear is associated with several of these, particularly feather pecking (Uitdehaag et al., 2009; de Haas et al., 2010; de Haas et al., 2013; Kops et al., 2013; Rodenburg et al., 2013), cannibalism (Newberry, 2004), and fear-induced smothering (Bright and Johnson, 2011; Barrett et al., 2014). The primary factors associated with problem behaviours were housing system and issues with climatic control. Farmers with aviary systems reported more problem behaviours compared to producers with furnished cages. These results correspond with other studies suggesting that aviaries are associated with a higher proportion of problem behaviours compared to furnished cages (Rodenburg et al., 2005; Tauson, 2005; Sherwin et al., 2010; Shimmura et al., 2010; Lay et al., 2011; Stadig et al., 2016). However, housing system per se is not necessarily the cause of the results in paper **IV**. The housing system may be confounded with factors that the questionnaire did not consider. For example, egg producers with furnished cage systems might be different from aviary egg producers in terms of their competence, motivation, level of awareness, or possibility to detect these problem behaviours. For instance, if eggs are laid outside a nest box in a furnished cage, the tilted floor will allow the egg to end up in the egg belt without increased labour on the part of the farmer. Also, consequences of problem behaviours in loose-housed systems often affect a larger number of individuals and are therefore easier for a farmer with an aviary to notice.

From an animal welfare perspective, comparing different housing systems is complicated (see section 1.2.3.1.). Birds housed in furnished cages often have lower mortality and lower prevalence of disease (EFSA, 2005; Fossum et al., 2009; Jansson et al., 2010; Lay et al., 2011; David et al., 2015a; David et al., 2015b). If one emphasises a biological functioning approach, furnished cages might, therefore, be considered superior to loose-housed systems. However, from a subjective experience approach, the limited possibility to perform strongly motivated behaviours (e.g. foraging, wing flapping, and dustbathing) in furnished cages (Widowski et al.,

2016) suggests that welfare is better in a loose-housed system. In fact, one of the main advantages of loose housing is that birds have the possibility to perform more behaviours compared to birds in furnished cages. Consequently, it will also be easier for aviary-housed birds to express problem behaviours. Without a more holistic welfare approach including a wider range of welfare indicators, the results in paper **IV** should not be considered evidence of aviaries being inferior to furnished cages. Rather, it is possible that the birds in furnished cages would have expressed the same level of problem behaviours if the cages did not restrict their behaviour.

The other main factor associated with problem behaviours in paper **IV** was issues with climatic conditions at the egg production farm. The aviary respondents reported more problems with climatic control compared to producers with furnished cages. This result supports other studies suggesting that furnished cage systems have better air quality (lower levels of dust and ammonia) compared to aviaries (Nimmermark et al., 2009; Lay et al., 2011; Le Bouquin et al., 2013; David et al., 2015a; David et al., 2015b). Uneven temperatures or suboptimal ventilation can influence the stocking density locally as birds choose to avoid certain areas but cluster in other parts of the house. The effect of bird movement on local stocking density and how this influences laying hen welfare is not entirely understood. Some, but not all studies report an increased risk of aggression and reduced feather quality with increased stocking density (Gunnarsson et al., 1999; Gunnarsson et al., 2000; Channing et al., 2001; Nicol et al., 2006; Collins et al., 2011; Widowski et al., 2016). Dysfunctional climatic control can also cause a build-up of gases such as ammonia and carbon dioxide. Ammonia exposure can cause respiratory problems for the birds and the stock people (Kirkhorn and Schenker, 2002; Kirychuk et al., 2003; David et al., 2015a; David et al., 2015b). In henhouses with cage systems, reduced ventilation was more commonly found in corners and along the back of the house. Consequently, the build-up of gases poses a potentially bigger problem for the stock people than for the animals (Prodanov et al., 2016). On the contrary, in loose-housed systems where the birds have access to the areas with build-up of gases, the gas concentrations might also be a concern for the laying hen welfare. The results in paper **IV** do not explain the causative relationship between issues with climatic control and the associated problem behaviours.

The association between issues with climatic control and increased occurrence of problem behaviours as perceived by the egg producers might be real. However, from the results in paper **IV**, we cannot exclude the possibility that climatic control is a reflection of the general

management. In other words, flocks that are poorly managed (which can contribute to poor climatic conditions) are more likely to show problem behaviours. Loose housing systems are considered more challenging to manage and demand a better stockmanship compared to confined housing systems (Appleby and Hughes, 1991; Häne et al., 2000). The effect of the stock people can, therefore, be of greater importance for loose-housed birds compared to cage-housed birds. As paper **IV** does not separate the effect of stock people or management from the climatic conditions, follow-up studies are needed to elucidate the association further.

For the furnished cage producers, the size of the flock was the only risk factor associated with the occurrence of problem behaviours. Fewer problem behaviours were reported by farmers with at least 7,500 birds compared to farmers with less than 7,500 birds. The time the farmer spent inspecting the birds was a maximum of 60 min irrespective of the number of birds per farm. Consequently, we cannot exclude the possibility that the egg producers in farms with more birds overlooked problem behaviours to a larger degree. Another possible explanation for this association is that a bigger farm could reflect a more dedicated and professional egg farmer compared to the producers with smaller farms.

In paper **IV**, none of the risk factors associated with producer-perceived problem behaviours in laying hens were rearing-related. This result was unexpected, as other studies have identified several rearing factors related to the occurrence of feather pecking and reduced feather quality during lay (Nicol et al., 2001; Bestman et al., 2009; Lambton et al., 2010; Gilani et al., 2013; Lambton et al., 2013). In an experimental study, 34% of the variation in plumage quality at 40 weeks of age was explained by the distance to a human and to high levels of severe feather pecking at five weeks of age (de Haas et al., 2014a). In a longitudinal study, 21 risk factors during rearing and 17 risk factors during lay were identified for the development of feather pecking (Gilani et al., 2013). It is plausible that the study design in paper **IV** hampered our ability to detect rearing-related risk factors. Originally, the study was meant to include an additional questionnaire for rearing farmers. However, the number of rearing farmers willing to participate in the study was too low, so we ended up only focusing on the egg producers. The rearing-related questions in the survey only included info about the name of the rearing farmer who provided the flock, the housing system at the rearing farm, and the age of the birds when they arrived at the egg production farm. In other words, the results in paper **IV** do not mean that problem behaviours are independent of factors during rearing. On the contrary, the

results from papers **I**, **II,** and **III** demonstrate that aspects of the rearing environment do affect fear responses and thereby probably also fear-related problem behaviours in adult laying hens.

5.4. Interpretation of the fear responses using Miller's model (paper I, II and III)

The fear tests used in papers **I**, **II,** and **III** are based on a behavioural assessment of the animals' conflicting motivations to approach and avoid fear-inducing stimuli. Referring to the model proposed by Miller (1944), aspects of the rearing environment may have affected the motivation to avoid (Figure 8) or the motivation to approach (Figure 9) the stimuli to which birds were exposed during testing. In papers **I** and **II**, the aviary-reared birds spent more time close to the stimuli compared to the cage-reared birds. In paper **III**, birds with early access to a pecking substrate spent more time close to the novel object compared to birds reared without a pecking substrate. Based on the work covered by this thesis, it is uncertain whether the more complex rearing environments reduced the motivation to avoid or increased the motivation to approach the stimuli. Similarly, the more barren rearing environments (cage rearing or lack of a pecking substrate during early life) either weakened the motivation to approach or intensified the motivation to avoid the stimuli. However, approach-related appetitive behaviour is related to activity in dopaminergic systems, and these were unaffected by the choice of housing system during rearing (Tahamtani et al., 2016b). It is therefore more likely that aviary rearing reduces fear than simply increasing appetitive responses (approach tendencies).
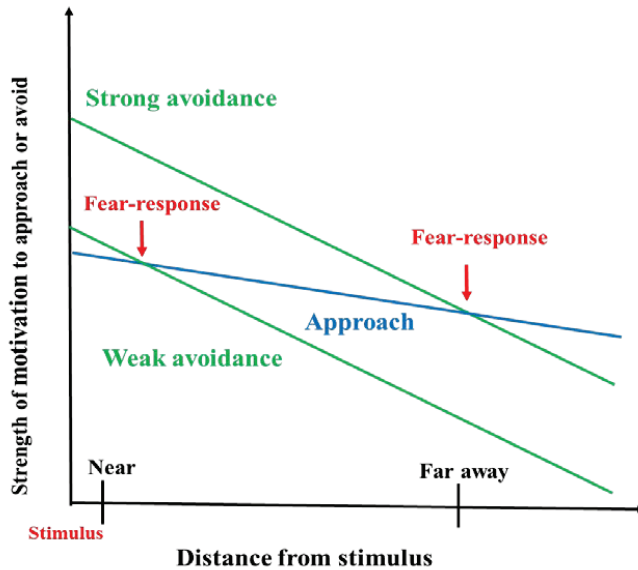
Figure 8. Illustration of how differences in the motivation to avoid the stimulus affect the fear response of the animal. Under the assumption that the motivation to approach is constant, a strong motivation to avoid the stimulus will cause the animal to stop further away from the stimulus. This could be the case of the cage-reared birds in papers **I** and **II** and the birds reared without a pecking substrate in paper **III**. In the same way, if the motivation to avoid is reduced, the animal will move closer to the stimulus before the motivation to avoid overcomes the motivation to approach the stimulus. This could be the case for the aviary-reared birds in papers **I** and **II** and the paper-reared birds in paper **III**. Modified from Miller (1944).
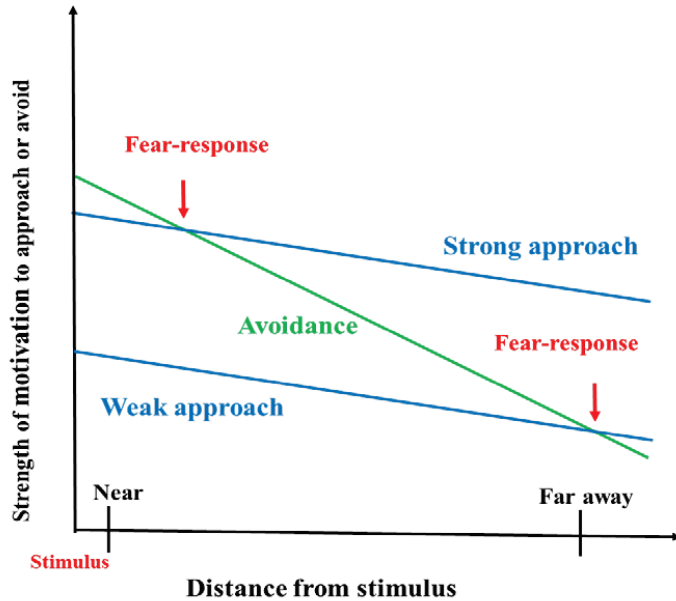
Figure 9. Illustration of how differences in the motivation to approach the stimulus affect the fear response of the animal. Under the assumption that the motivation to avoid is constant, a weak motivation to approach the stimulus will cause the animal to stop further away from the stimulus. This could be the case for the cage-reared birds in papers **I** and **II** and for the birds reared without a pecking substrate in paper **III**. In the same way, if the motivation to approach is strong, the animal will move closer to the stimulus before the motivation to avoid overcomes the motivation to approach the stimulus. This could be the case for the aviary-reared birds in papers **I** and **II** and the paper-reared birds in paper **III**. Modified from Miller (1944).

As introduced in section 1.4, fearfulness is the underlying predisposition of an individual to be easily frightened (Boissy, 1995). Scientists disagree whether one fear test is sufficient to assess animals' fearfulness. Some argue that fear responses are stimulus-specific and vary with different test situations (Miller et al., 2005; 2006; Mazurek et al., 2011). Following the latter view, the result of a single test might not be accurate, and a variety of fear tests may be required to evaluate the fearfulness of the animal (Waiblinger et al., 2006). Consequently, the results from the novel object test in paper **I** may not necessarily represent an accurate estimate of the underlying fearfulness. However, if results obtained from different fear tests correlate, it is likely that the results reflect the underlying fearfulness of the birds. Work on laying hens has indicated that behavioural responses in tonic immobility tests and variations of open field tests, or novel environment tests, correlate closely (Suarez and Gallup, 1981; Forkman et al., 2007). The principal component analysis conducted in paper **II** generated one component that was suitable for further analysis. All the outcome variables from the novel object test and the

voluntary human approach test correlated and loaded on this component. Individuals with high scores on this component were inactive, spent a considerable amount of time standing still and spent little time close to the human or novel object. The results in paper **II** correspond well with a similar study where standing alert and locomotion loaded on the same principal component (Campler et al., 2009). Also, inactivity is considered as an indicator of fearfulness in laying hens (Forkman et al., 2007). The component score in paper **II** is, therefore, presumably a better estimate of the underlying level of fearfulness compared to the results from the single fear test used in paper **I**, which may be more stimulus-specific.

In paper **III**, the early provision of a pecking substrate reduced the fear response towards the novel object, but not towards the stationary person. Interpreting these results as indicators of the animals' fearfulness is, therefore, more difficult. The results from paper **III** could indicate that the provision of a pecking substrate reduced the fear of novelty rather than the underlying level of fearfulness. However, as the assessment in paper **III** was on flock level rather than on individual level, correlation analysis of the behavioural measures could not be conducted. Therefore, paper **III** does not necessarily answer the question of whether the provision of a pecking substrate during rearing affects the underlying fearfulness or only the specific response to a novel object.

5.5. Methodological considerations

All the studies presented in this thesis relied on the participation and cooperation of commercial rearing farmers and egg producers. Commercial rearing farmers reared the birds included in papers **I**, **II**, and **III**. This was our only option, as we did not have access to experimental facilities that could simulate an environment and management similar to commercial rearing conditions. On one hand, this is positive as it increases the generality of the obtained results compared to results solely based on experimental studies (Dawkins, 2012; Gilani et al., 2012). On the other hand, by allocating the rearing to the rearing farmers, we had limited control of the environment and management routines. For example, the amount and quality of human contact birds receive during early life is of importance to the birds' fear responses (Jones, 1996). Human contact during rearing, and the strength of positive or negative emotions involved in the perception of humans, possibly affected the results obtained from the fear tests involving

humans (papers **II** and **III**) (Jones and Waddington, 1993; Jones, 1993; 1994; 1995; Waiblinger et al., 2006; Graml et al., 2008a).

Furthermore, assigning the administration of the treatments and the distribution of the birds to the farmers reduced the researchers' control and introduced opportunity for errors. In paper **III**, all the Norwegian rearing farmers who were willing to participate were first visited to assess whether their rearing houses were suitable to include in the study. If so, the rearing farmers were instructed on how to split the aviary rows into paper-reared chicks and chicks reared on wire mesh. The importance of separating the groups after release into the aviary corridors, and also when collecting birds to distribute to different egg producers, was emphasised. Due to practical and economic constraints, it was impossible to visit the rearing farms a second time during the study. Instead, the researchers were available via phone/email to answer any questions the rearing farmers might have. 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, mixing birds when sending them to producers, and inaccurate bookkeeping. Also, some of the egg producers did not allow the researchers to have access to the animals to perform the data collection. These issues resulted in substantially fewer flocks being included in the final dataset in paper **III**. Despite the fact that a total of eleven rearing farmers were visited at the beginning of the study, data from only five rearing farmers remained after exclusion of flocks that did not conform to the instructions. Similarly, although 47 egg producers were visited and generated data, only 23 of the flocks remain in the final dataset. These numbers illustrate some of the difficulties and the considerable demand on resources when performing experiments under commercial farming conditions.

In paper **II**, the housing and management of the birds after transfer from the rearing farm occurred at the NMBU research facilities. This enabled standardisation and control of factors that could influence the outcome of the fear tests (e.g. uniform stocking density, optimisation of light intensity, controlled amount of exposure to humans). On the contrary, data collection for papers **I** and **III** were performed at commercial egg production farms rather than at designated research facilities. In these studies, we lacked the opportunity to ensure the same level of control and standardisation as in paper **II**. The fear tests were either conducted while the birds were in groups in their home environment (papers **I** and **III**) or isolated in a separate arena (paper **II**). The performance of the fear tests in the home environment limits confounding effects of exposure to novelty and reduces stress due to social separation (Waiblinger et al.,

2006). Also, the behavioural observations in paper **I** were based on video recordings, alleviating the potential confounding effect of fear caused by human presence. Nonetheless, we cannot exclude the possibility that the results obtained in the fear tests in the home environment (papers **I** and **III**) were influenced by the presence of other birds (Waiblinger et al., 2006). When conducting the fear tests in the aviary corridors (paper **III**), several factors related to the egg production farm might have influenced the results. The size of the house relative to the flock size (i.e. stocking density), the width of the corridors where the tests were performed, the light intensity inside the henhouse, and the presence of barriers influenced the ease with which the birds could approach or avoid the human during the fear test. For example, in aviaries where the light intensity was high, there is no doubt in that the birds saw the experimenters. In other houses, the light was so dim that it appeared as though the birds did not notice the observer. In these cases, it was impossible to discriminate between birds that were passive because they were unaware, due to the low light intensity, and those that were less fearful. On the contrary, the novel object test was less influenced by the light intensity in the henhouse as the object was always placed close to a light source on the ground. Similarly, some of the aviaries were built to contain bigger flocks than the current legislation allows, and thus the birds had plenty of space. In these situations the birds had the option to avoid the experimenters, not necessarily because they moved away when approached, but just because they happened to be positioned in other parts of the house while the stationary person tests were performed. A major disadvantage when comparing fear responses in different housing systems is that tests that apply to one type of housing system might not be comparable to tests that are feasible in a different housing system. Consequently, it might be better to perform fear tests in a standardised arena. A study comparing fear responses when exposed to fear tests in the home environment or a separate arena concluded that conducting the fear tests in the arena was the best option to assess fearfulness of the laying hens (Graml et al., 2008b). However, fear tests in a separate (novel) arena introduces confounders due to capture, handling and social separation (Waiblinger et al., 2006; Forkman et al., 2007). In paper **II** we tried to control for the effect of capture and handling by limiting the time from capture in the home pen to starting the test. Besides, we balanced the order of testing across room, pen, and treatment group. However, we were not able to include measures indicative of social separation in the analysis of the behaviour. Motivation for social reinstatement was a potential confounder that might have affected the results in paper **II**.

The data collection in paper **III** was based on the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009) and on papers evaluating fear tests in loose housing systems (Raubek et al., 2007; Graml et al., 2008a; Graml et al., 2008b; de Haas et al., 2014a; de Haas et al., 2014b). However, after the first three farm visits, we realised that hardly any birds came within the required 25 cm of the experimenters' feet. We therefore considered it necessary to include an additional measure of birds that approached within 2 m of the experimenters. By doing so, most flocks had birds that entered the designated area. It is possible that the rearing treatment did affect the birds' response to the human but that our chosen measures of 2 m and 25 cm were improperly calibrated to distinguish the paper-reared birds from the birds reared without a pecking substrate.

During the design stage of the experimental studies in this thesis (papers **I**, **II**, and **III**), power calculations for sample size were performed using JMP® version 11.1.1 (SAS Institute Inc., Cary, NC, USA). Alpha was set to 0.05, the standard deviation to 1, the difference to detect to 1.5 standard deviations, and the minimum acceptable power required as 80%. With these numbers, it was determined that the sample size would be a minimum of nine flocks per treatment group. However, within the allocated time and budget, the sample sizes ensured power between 90% and 100%. In other words, the results presented in papers **I**, **II**, and **III** are considered to be robust and trustworthy.

The data collection in paper **IV** relied on farmers' undertaking a questionnaire. This method possibly introduced systematic errors. First of all, the egg producer is an unmeasurable confounder (Dohoo, 2014) as he or she both affects the exposure (i.e. management of the flock) and the outcome (i.e. is the one who reports the behaviours). Furthermore, the questionnaire is sensitive to information bias (e.g. recall bias, misinterpretation of the questions, or different opinions about the provided definitions of the behaviours) (Dohoo, 2014). Finally, we cannot rule out the possibility of selection bias due to non-response bias (Dohoo, 2014) as we did not follow up the non-responders to see whether their replies would have differed from those of responders. Non-response bias might have contributed to higher reports of problem behaviours, as producers without experience with these issues might have lacked the motivation to complete the questionnaire. Non-response bias could have also resulted in under-reporting if producers experiencing these problems did not reply to the questionnaire. However, the aim of paper **IV** was to assess risk factors rather than detect the prevalence of problem behaviours. On

the contrary, estimating the prevalence of problem behaviours in Norwegian layer flocks would have required a different study design based on random selection of egg producers.

5.6. Conclusions

The work presented in this thesis aimed to increase knowledge on how certain aspects of the rearing and production periods affect fear responses in adult laying hens. Under commercial egg production, fear is associated with behaviours such as feather pecking, cannibalism, and fear-induced smothering. These behaviours compromise laying hen welfare, both from the biological function approach and a subjective experience approach. Measures of fear are therefore relevant welfare indicators in laying hens.

The literature suggests that genetics and a range of environmental factors affect fear responses. Environmental complexity is predominantly dependent on the housing system. Papers **I** and **II** aimed to test whether cage rearing versus aviary rearing affected fear responses in adult laying hens. The cage-reared birds spent less time close to a novel object compared to the aviary-reared birds. In paper **II**, the cage-reared birds spent less time close to a novel object or a human and were less active compared to the aviary-reared birds. Combined, the results of papers **I** and **II** indicate that rearing in a more complex aviary environment reduces fear responses compared to rearing in a barren cage environment. The results might suggest that the rearing environment not only affects the fear response towards a specific novel object (paper **I**) but also the birds' underlying fearfulness (paper **II**). Paper **IV** identified an association between the housing system used during the production phase and the occurrence of problem behaviours. Egg producers with aviary systems reported more problem behaviours than those with furnished cage systems. From a welfare perspective, this could indicate that welfare is better in furnished cages. However, the questionnaire investigated associations rather than causal relationships. Moreover, the questionnaire relied on farmer observation and interpretation of fear-related behaviours. Whether the difference in problem behaviours is real or was influenced by unmeasurable confounders is uncertain.

In addition to housing system, the amount and complexity of stimuli experienced by the birds can be further increased by the provision of environmental enrichment. Inanimate objects, as well as sawdust and edible supplements, encourage activity, exploration, and foraging

behaviour. Paper **III** aimed to test whether the provision of a pecking substrate from the first day of life reduced fear in adult laying hens under commercial conditions. As predicted, more birds reared with access to a pecking substrate approached a novel object at 30 weeks of age compared to birds reared without a pecking substrate. However, the rearing effect on fear responses was only detected as long the egg producer did not provide environmental enrichment. In other words, provision of environmental enrichment at the egg production farm can counteract a lack of access to a pecking substrate during the early rearing period. The results of paper **III**, therefore, emphasise that environmental complexity, not only during rearing but also during the egg production phase, affects fear responses in laying hens. In contrast to paper **III**, paper **IV** did not identify the provision of environmental enrichment at the egg production farm as associated with the farmer-reported occurrence of problem behaviours. In paper **IV**, hardly any producers answered no when asked if the birds were provided with environmental enrichment. Therefore, we were not able to include this parameter in the statistical analysis.

In conclusion, the results from papers **I**, **II**, and **III** suggest that increased environmental complexity during rearing reduces fear responses in adult laying hens. During rearing, the housing system is the primary determining factor (papers **I**, **II**, and **IV**), but within aviary rearing systems, the addition of a pecking substrate can further increase the environmental complexity (paper **III**) and reduce fear. At the production farm, the housing system is possibly the principal determining factor in the level of environmental complexity experienced by the birds (papers **III** and **IV**). However, the addition of environmental enrichment at the egg production farm can increase the level of stimulation even further (papers **III** and **IV**). Complete elimination of fear is neither desirable nor required to safeguard the best welfare possible for laying hens in commercial egg production. But by increasing environmental complexity and stimuli experienced by the chicks during rearing, and by providing environmental enrichment and ensuring optimal climatic conditions during the production phase, farmers can improve the welfare of laying hens in commercial egg production.

5.7. Some future research needs

Papers **I** and **II** only included observations of fear responses until 23 weeks of age. This thesis did not address the effect of early environmental complexity on fear responses towards the end

of lay. It has been suggested that fear increases over time in cage-housed adult birds (Brake et al., 1994). Higher mortality rates have also been reported among aviary-reared birds in furnished cages compared to cage-reared birds (Tahamtani et al., 2014). To follow up the results in papers **I** and **II**, a longitudinal study with additional measures of fear responses until the end of the production phase would be desirable. Based on the types of enrichment observed by the researchers (paper **III**) and reported by the egg producers (paper **IV**), it seems that most egg farmers use traditional types of enrichment such as shell sand, pecking blocks, and pieces of plastic. Few farmers mentioned the use of auditory enrichment such as playing the radio. Auditory enrichment is another practical way to enrich the environment and is associated with reduced fear responses in laying hens (Jones, 2004; Davila et al., 2011; de Haas et al., 2014a). More research on the use of auditory enrichment (e.g. human voices versus music) would be needed to give appropriate advice to commercial rearing farmers and egg producers.

During conversations with the rearing farmers, none of them said they provided the pullets with environmental enrichment during rearing. Only a few rearing farmers scatter sparse amounts of sawdust in the corridors before releasing the birds from the aviary rows; the majority rely on the accumulation of dust as 'pecking substrate'. Based on the findings in paper **III**, indicating that the provision of something as simple as a thin layer of paper from the first days of life reduced fear responses, it can be argued that birds benefit from increased exposure to environmental enrichment from an early age. In this context, it would be valuable to assess whether pullets differ from laying hens in what they find 'enriching' (Jones, 2004).

Based on the results of paper **IV**, future research is required to investigate a possible cause-effect relationship between issues with climatic control and problem behaviours. One possibility would be a follow-up cohort study at the farms that reported problems with climatic control. Such a study should ideally include objective measures of gas concentrations, airflow, temperatures, and air humidity in different parts of the house. Additionally, it would be of interest to link the occurrence of difficulties with climatic conditions with the age of the henhouse. Older buildings may be a risk factor, for example due to insufficient ventilation. It could be expected that more recently built houses would be better capable of maintaining good climatic conditions. This information could be useful for producers considering changing their housing systems in the future.

When comparing the observations of producers with those of trained researchers, it has been found that producers underestimate the amount of feather pecking (Huber-Eicher and Sebo, 2001; Lambton et al., 2010). In this context, it would be beneficial to assess how the perception of other problem behaviours compares between farmers, researchers, veterinarians, and poultry advisers.

Furthermore, the results in paper **IV** indicate that more research is required to improve the administration of litter as well as access to and use of the dustbathing area in furnished cages. Finally, paper **III** assessed the effect of a pecking substrate on fear responses only when the birds were housed in aviary systems. It would be interesting to conduct a similar experiment focusing on birds in furnished cages.

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Appendices I - IV

Paper I

Rearing Laying Hens in Aviaries Reduces Fearfulness following Transfer to Furnished Cages

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Appropriate rearing is essential for ensuring the welfare and productivity of laying hens. Early experience has the potential to affect the development of fearfulness. This study tested whether rearing in aviaries, as opposed to cages, reduces the fearfulness of laying hens after transfer to furnished cages. Fear responses were recorded as avoidance of a novel object in the home cage. Lohmann Selected Leghorns were reared in an aviary system or conventional rearing cages and then transported to furnished cages at 16 weeks, before the onset of lay. Observations of a selection of birds were conducted at 19 ($N = 50$ independent cages) and 21 ($N = 48$ independent cages) weeks of age. At 19 and 21 weeks, cage-reared birds showed higher levels of fearfulness indicated by spending more time away from the novel object compared to aviary-reared birds. These results suggest that rearing in an enriched aviary environment reduces fearfulness up to the fifth week after transfer to a new housing system, compared to rearing in cages.

Keywords: laying hens, chicken, welfare, rearing, development, fearfulness, stress, fear

INTRODUCTION

Under natural conditions, fear normally functions to protect animals from dangerous situations, and thereby increases their chances of survival (1). However, under production conditions, exaggerated fear is a potent stressor associated with activation of the hypothalamic–pituitary–adrenocortical axis. Fear may have negative consequences for animal welfare and productivity if the fear responses are exaggerated, inappropriate, or expressed in a restrictive environment (2–5). Fearfulness is the predisposition of an individual to be easily frightened (1, 6) and is influenced by both genetic (7) and developmental factors.

The early environment may have a great impact on the development of fearfulness and associated activation of the hypothalamic–pituitary–adrenocortical axis in response to stressors (6, 8–11). Exposure to increased environmental complexity during rearing has been found to reduce fearfulness during adulthood in several species, including mice (12), pigs (13), and chickens (14). For laying hens, the housing system during rearing is a major source of environmental variability, illustrated by the large difference between the cage and aviary-rearing systems. However, few studies have tested for effects of the rearing system on later fearfulness in laying hens. A study comparing floor-housed adult birds reared on sand, straw, or wire from 0 to 4 weeks found that birds reared on wire were the most fearful, as indicated by longer durations of induced tonic immobility (14).

A study comparing cage-housed adult birds reared in a floor or cage system found that floor-reared birds were more active, and displayed more flighty responses to a human than cage-reared birds (3). However, a similar study failed to find differences in escape or tonic immobility responses between floor and cage-reared laying hens housed in cages as adults (15). Other studies that have tested for effects of exposure to varying degrees of environmental complexity confound effects of rearing and housing of adult birds [see Ref. (16, 17)]. To the best of our knowledge, there are no previous studies comparing effects of rearing in a complex aviary system with rearing in a barren cage environment on fear responses in birds housed in the same environment as adults.

There is consensus that an individual's fearfulness can be quantified by observing its response to novelty (6, 18–20). Novel object tests measure the conflicting motivations to approach and avoid a novel object, as described by Miller's Model (21, 22). This model states that the animal will approach a novel object up to the point at which the motivation to avoid the stimuli becomes as strong as the motivation to approach it (21–24). The duration of time spent in proximity to a novel object can, therefore, be used to quantify an animal's fearfulness.

The aim of this study was to test the hypothesis that birds reared in a conventional rearing cage system would be more fearful when exposed to a novel object during the production period than birds reared in a complex aviary system. The hypothesis was tested in a controlled experimental study where laying hens were reared in either enclosed cages or an aviary system and then transferred at 16 weeks of age to the same house containing furnished cages. Fear responses were evaluated at 19 and 21 weeks of age using the duration of time spent close to a novel object in the home cage. The birds used in the present study are identical to those used by Tahamtani et al. (25). The previous article focused on using undisturbed comfort behavior and alertness in response to a novel object as indicators of animal welfare and not specifically as indicators of fearfulness. Production data, mortality, and blood glucose levels were also presented in the previous article. The current study is conceptually unique in focusing on fearfulness as indicated by approach–avoidance behavior.

MATERIALS AND METHODS

General Description of Subjects and Rearing Conditions

As previously mentioned, the subjects and most of the methods in the current study correspond to Tahamtani et al (25). However, the methods and results for approach–avoidance behavior are unique. Non-beak trimmed, female Lohmann Selected Leghorn chickens (*Gallus gallus domesticus*) of ages 0–21 weeks and normal health status were used in this study within a commercial setting. These birds were hatched and reared in one of two rearing treatments: an aviary- or a conventional cage-rearing system. All eggs originated from the same flock and were incubated at the same time by the same hatchery. 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. Rearing cages measured 6050 cm² and contained 17 birds

per cage (Housing Type: Big Dutchman Universa), giving a stocking density of 28 birds/m². The flooring in these cages was wire, and no bedding was provided. The density of birds in the aviary-rearing system (Housing Type: Big Dutchman Natura Rearing) was 24 birds/m². The bedding on the floor of the house was sawdust (small dimension wood shavings). Pullets were provided with *ad libitum* access to feed using a chain dispersal system. The feed type was conventional pullet feed produced and sold by Felleskjøpet, Norway. The diets used were "oppdrett 1" for 0- to 7-week-old birds and "oppdrett 2" for 8- to 17-week-old birds. The nutritional content is optimized for layers of this age according to recommendations by Lohmann (26).

At 16 weeks, the birds from both housing systems were transported to a single farm. The housing at the farm was furnished cages (Housing Type: AVIPLUS, Big Dutchman, designed for housing 10 hens according to EU requirements), measuring 63 cm × 120 cm (7560 cm²) and containing between eight and nine birds per cage according to Norwegian legislation. A total of 7500 birds, half of which came from each rearing treatment, were included in the study. The composition of a group was not mixed, cages either contained birds reared in conventional rearing cages or birds reared in the aviary system. The furnished cages included access to dustbathing substrate (a small amount of crushed feed in a 1200 cm², oblong litter bath), a nest box, and two perches. The cages were tiered within the house creating three levels of cages, and arranged in four rows. Each row either contained aviary- or cage-reared birds, allowing birds from different rearing treatments to see one another across an adjacent aisle. The farm operated on a light cycle that was altered according to recommendations by Lohmann (26). During the period of behavioral observations, the light in the chicken house turned on at 07:00 and turned off at 16:00. Feed was provided *ad libitum* using a chain dispersal system in a feeding trough at the front of the cage and water was provided *ad libitum* by nipple drinkers (two per cage).

Data Collection

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. Both visits involved the collection of video footage from a selection of cages. A total of 50 furnished cages were recorded at 19 weeks of age, of which 28 contained aviary-reared birds and 22 contained cage-reared birds (see **Table 1**). At 21 weeks of age, a total of 48 furnished cages were filmed, of which 24 contained aviary-reared birds and 24 contained cage-reared birds. The videos were collected on two consecutive days between the times of 09:00 and 14:00 (see **Table 1**). The number of cages per tier at the different ages is shown in **Table 1**. Cage was used as the statistical unit. Cages were selected to represent all areas of the house (row and tier). Different cages were filmed on each farm visit to avoid effects of the first observation upon the second. Two cages from each treatment were filmed concurrently to balance the treatments in case of time effects. After recording had begun, the researcher left the house. Hand-held cameras (Everio, JVC) mounted on tripods were set up, so that the frontal aspect of the cage was filmed. Ten minutes after filming was started, a researcher returned to add the novel objects to the cages. The novel objects used were empty plastic bottles, hung with a wire attachment on

TABLE 1 | Overview of data showing the number of cages per treatment per test day and number of cages per treatment per tier at 19 and 21 weeks of age.

Age	19 weeks			21 weeks		
Test day	Day 1	Day 2	Total	Day 1	Day 2	Total
Aviary reared	15	13	28	12	12	24
Cage reared	10	12	22	12	12	24
Total	25	25	50	24	24	48

Age	19 weeks				21 weeks			
Height of cage	Tier 1	Tier 2	Tier 3	Total	Tier 1	Tier 2	Tier 3	Total
Aviary reared	8	10	10	28	6	5	13	24
Cage reared	8	6	8	22	5	12	7	24
Total	16	16	18	50	11	17	20	48

the front bars of the cage so that the bottle was just inside the cage approximately 10 cm from its right boundary. The right side of the cage in front of which the novel object was placed contained a nest box, the roof which was the litter area. The researcher then left the room containing the birds and recording continued for a further 10 min. Subsequently, the researchers returned to remove the novel objects and the cameras and assembled them in a different location within the house. Footage collection continued in this manner until the required number of cages was filmed.

Novel Object Test

Observer XT 7.0 software (Noldus Information Technology, Wageningen, The Netherlands) was used for behavioral analysis of the footage. The behavioral analysis was conducted on the basis of video recordings by a single researcher who was blind to the rearing background of the birds. When this analysis was started, the cage was divided into four equal zones of increasing distance to the novel object from right to left, using marks on the screen (zone 1 contained the novel object; zone 4 was furthest away from the novel object). Observations commenced 1 min after placement of the novel object into the home cage and measured the duration of time a focal bird spent in the different zones after introducing the novel object. One bird per cage was used. The observation was subsequently continued for 8 min. Before beginning the observation at the time of video-based analysis, a focal subject was selected in the following manner: the video was paused at the start of the observation. Chickens were numbered from left to right, and a bird selected randomly. In the event of the focal subject's movement out of view of the camera, the protocol for reselection was to observe the bird at the closest proximity and in front of the previous focal bird (closest to the front of the cage), to avoid influencing the duration of occupation in any given zone. Behaviors were coded in such a way that any one code represented the zone of occupation (proximity to novel object). Behavior was recorded continuously, and the durations of time spent in each zone were mutually exclusive.

Statistical Analysis

The ANOVA model that was used included the factors rearing treatment, tier (top, middle, or bottom), and the interaction between treatment and tier. Day was initially included in the model as a fixed factor but was removed from the model as it did not have any effect. All factors were fixed. Results for ANOVA are presented as F values and p values. Means and SD are presented for the raw, untransformed data. The duration of times spent in the 1/4, 1/2, and 3/4 of the cage closest to or containing the novel object were used as indicating proximity to the novel object. If needed, these variables were Box–Cox transformed to meet the assumptions of a GLM. Data for the two ages were analyzed separately. All statistical analysis was performed using JMP version 11.0 (SAS Institute Inc., NC, USA).

Ethical Statement

After reading a detailed formal application for permission to perform this field study (application ID 3868) the Animal Research Authorities ("Forsøksdyrutvalget," Norwegian Food Authority, Norwegian Government) stated that no specific permission was needed for the activities described in this study. The rearer had previously received permission from the Norwegian Food Authority to rear birds in traditional rearing cages. Following the study, the birds continued to be housed for egg production purposes until their euthanasia at 76 weeks of age. The study did not involve endangered or protected species.

RESULTS

Upon introduction of the novel object, birds typically vocalized and fled to the opposite end of the cage, but these responses were not scored systematically or quantified.

Data from Visit at 19 Weeks of Age
Duration of Time Spent in Zone 1
For the duration of time spent in zone 1, in which the novel object was situated, there was no effect of treatment ($F_{1,44} = 2.6227$; $p = 0.1125$), tier ($F_{2,44} = 0.3290$; $p = 0.7214$), or the interaction between them ($F_{2,44} = 0.5828$; $p = 0.5626$).

Duration of Time Spent in Zone 1–2
When combining the time spent in zone 1 and 2, the aviary-reared birds tended to spend more time closer to the novel object compared to the cage-reared birds ($F_{1,44} = 3.0103$; $p = 0.0897$; aviary-reared: 248.071 ± 137.87 (mean $\pm$ SD) s; cage-reared: 170.045 ± 147.285 s).

The tier ($F_{2,44} = 0.8479$; $p = 0.4352$) and the interaction between tier and treatment had no effect ($F_{2,44} = 1.8932$; $p = 0.1627$).

Duration of Time Spent in Zone 1–3

The aviary-reared birds spent more time close to the novel object compared to the cage-reared birds ($F_{1,44} = 5.6105$; $p = 0.0223$; aviary-reared: 405.857 ± 89.361 s; cage-reared 340.273 ± 112.877 s). The tier did not affect the amount of time spent close to the novel object ($F_{2,44} = 0.3187$; $p = 0.7287$). The duration of time close to the novel object had a tendency to be influenced by the interaction between treatment and tier ($F_{2,44} = 2.8072$; $p = 0.0712$). Aviary-reared birds in furnished cages at the top tier tended to spend more time close to the object compared to cage-reared birds housed on the lowest tier.

Data from Visit at 21 Weeks of Age
Duration of Time Spent in Zone 1

The aviary-reared birds spent more time close to the novel object compared to the cage-reared birds ($F_{1,44} = 5.2791$; $p = 0.0267$; aviary-reared: 48.083 ± 52.531 s; cage-reared 46.375 ± 88.763 s). Tier affected time spent in zone 1 ($F_{2,44} = 4.3217$; $p = 0.0196$), with birds from the middle tier spending more time (79.176 ± 98.420 s) close to the novel object than birds from the bottom tier (33.273 ± 54.019 s) and the top tier (27.75 ± 41.974 s). There was no interaction between treatment and tier ($F_{2,44} = 0.3753$; $p = 0.6893$).

Duration of Time Spent in Zone 1–2

When combining the time spent in zone 1 and 2, there was no effect of treatment ($F_{1,44} = 0.0005$; $p = 0.9831$), tier ($F_{2,44} = 2.1018$; $p = 0.1349$), or the interaction between them ($F_{2,44} = 1.1296$; $p = 0.3328$).

Duration of Time Spent in Zone 1–3

When combining the time spent in zone 1–3, there was no effect of treatment ($F_{1,44} = 0.0595$; $p = 0.8084$), tier ($F_{2,44} = 0.2979$; $p = 0.7439$), or the interaction between them ($F_{2,44} = 0.1847$; $p = 0.8320$).

DISCUSSION

This study tested the hypothesis that birds reared in a cage system are more fearful, when exposed to a novel object during the production period, than birds reared in an aviary system. This was supported by results that suggest rearing in a relatively complex aviary system reduces fearfulness in laying hens compared to rearing in a barren cage environment. The observation that aviary-reared birds had a greater duration of time spent in the 3/4 of the cage closest to the novel object at 19 weeks of age and a greater duration of time spent in the 1/4 of the cage closest to the novel object at 21 weeks indicates lower fearfulness in aviary-reared than in cage-reared birds. The treatment effects at the two ages were thus dependent on the definition of proximity to the novel object that was used, suggesting that *a priori* definitions of approach and avoidance may confer disadvantages. In addition

to effects of rearing, birds from the middle tier spent more time in the 1/4 of the cage closest to the novel object at 21 weeks of age than birds housed at the bottom or top tiers. The effect of tier may result from differences in the degree of exposure to caretakers as birds in the second tier have the closest proximity to humans during daily inspections. If so, the effect on the response to the novel object may also suggest some generalization of responses from humans to novelty.

Some studies have aimed at testing for effects of rearing conditions on later fear responses in adult laying hens. These are, however, difficult to compare to the present study because adult birds were not transferred to the same type of housing conditions after the rearing period [see Ref. (16, 17)]. This means that birds' fear reactions may be influenced by rearing but are also likely to be a product of the environment in which they are housed during the time they were observed and tested. To the authors' knowledge, no previous studies have used an experimental design that does not confound effects of rearing conditions with the housing of adult birds. Furthermore, none of the previous studies compared the effects of rearing in aviaries and cages. Several previous studies do, however, compare rearing in barren cages on wire to rearing on more complex substrates, such as sand, straw (14), or standard litter (3, 15). For the sake of comparison, one can arguably consider environments containing sand, straw, and other substrates to represent a higher degree of environmental complexity than barren cages with a wire floor. If one accepts this premise, increasing environmental complexity during rearing increases active reactions to handling and human presence (reduces the duration of tonic immobility) in birds whether they are housed in a floor system (14) or cages as adults [increased expression of flighty responses (3)]. The present study contributes new knowledge by showing that rearing in a more complex environment reduces the birds' avoidance of novel, fear-inducing stimuli. Because avoidance is one of the most fundamental characteristics of fear responses, this study is the first to indicate that exposure to increased environmental complexity during rearing reduces fearfulness in adult laying hens at three and five weeks following transfer to a furnished cage system.

Reduced approach and increased avoidance tendencies lie at the core of most operational definitions of increased fearfulness. There are, however, other factors that may also influence fear responses in the present study. On the introduction of the novel object test, a bird could start in the area farthest from the novel object or closest to the novel object and simply stay there if it was unresponsive and inactive. A responsive but inactive bird initially close to the novel object could flee to the area farthest from the object and then stay there. A responsive and active bird could flee to the area farthest from the object and but then continue to move around through the area close to the object afterwards. Also, we cannot exclude the possibility that group dynamics influenced individual fear responses. In the present study, it is not possible to disentangle these potential interacting effects related to responsiveness, activity, and group dynamics. They could all theoretically be confounded with fearfulness and are likely to contribute to residual variation in the data.

The study by Tahamtani et al. (25), using birds that were identical to those used in the current study, indicated that

aviary-rearing resulted in birds that displayed more comfort behavior at 19 weeks of age but had higher mortality throughout the production period. Higher expression of comfort behavior in aviary-reared birds suggests that they had better welfare, whereas the greater mortality throughout production suggests the opposite. The findings in the present study correspond well to the previously documented effect on comfort behavior but stand in contrast to the effect on mortality. Our results contradict the interpretation of Anderson and Adams (3), who suggested that birds showing active escape attempts (floor-reared birds) are more fearful than birds showing more passive responses (cage-reared birds). However, these researchers used test conditions (a human stimulus) and categorized behavior (from "calm, no nervous or evasive action" category 0 to "extreme escape and avoidance behavior" category 4) using an approach that was rather different than we used in the current study. Because observations were based on a combination of qualitative measures, they are difficult to interpret. Furthermore, results by Brantsæter et al. (27) indicate that flight responses when suddenly exposed to novel stimuli may not be related to fearfulness in laying hens but are more likely to reflect coping style.

Because observations were only carried out at 19 and 21 weeks of age but not later, the present study may not have clear implications for bird welfare in the long term. However, the treatment effect found at 19 weeks of age corresponds to a time at which birds are dealing with hormonal changes associated with the onset of lay [see Ref. (28)]. The positive effect of rearing in aviaries could, therefore, be important from an animal welfare perspective. The current study focused on the early production phase soon after transfer to the production facility. Future research could test whether the effect of rearing persists to later stages of development, especially given the previously reported negative effects of aviary rearing on mortality throughout the production phase (25). Farmers are unlikely to use aviary rearing as a method of reducing fearfulness in laying hens specifically, especially if they should later be used for producing in cages. It would, therefore, be useful to test effects of measures for reducing fearfulness that could be used in practice for conventional laying hens.

AUTHOR CONTRIBUTIONS

MB analyzed the data and wrote the first draft of the article; FT analyzed the data; RM collected data; TH collected data; RO scored behavior; CN contributed to conception and design of the study; AJ led the project, participated in conception and design of the study and collected the data. All authors contributed in writing the manuscript and approved the final version.

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Paper II

Exposure to Increased Environmental Complexity during Rearing Reduces Fearfulness and Increases Use of Three-Dimensional Space in Laying Hens (*Gallus gallus domesticus*)

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The complexity of the rearing environment is important for behavioral development and fearfulness. The aim of this study was to test the hypothesis that laying hens reared in a complex aviary system with exposure to mild intermittent stressors would be less fearful, less sensitive to stress, and would use elevated areas of the pen more often as adults than hens reared in a barren cage environment. Laying hens ($N = 160$) were housed in the same rearing house; half of the birds ($n = 80$) in an aviary and the other half ($n = 80$) in cages. At 16 weeks of age, the birds were transported to the experimental facilities. Their behavior was recorded at 19 and 23 weeks of age and analyzed by analysis of variance on individual scores for a fearfulness-related principal component generated using principal component analysis. The results indicate that aviary-reared birds have lower levels of fearfulness compared with cage-reared birds both at 19 weeks and at 23 weeks of age. When comparing the response induced by initial exposure to a novel object at 19 and 23 weeks of age, more aviary-reared birds tended to fly up at 19 weeks compared to the cage-reared birds, indicating a tendency toward a more active behavioral response in the aviary-reared birds than in cage-reared birds. There was no difference between treatments in the flight response at 23 weeks. The groups did not differ in defecation frequency or the concentration of fecal corticosterone metabolites at either age. At 19 weeks, observation of the spatial distribution in the home pens indicated that more aviary-reared birds spent time on the low perch, the elevated platform, and the upper perch, compared to the cage-reared birds. However, at 23 weeks of age, these differences were no longer detected. The results of this study support the hypothesis that increased environmental complexity during rearing reduces fearfulness of adult laying hens.

Keywords: laying hen, chicken, fearfulness, fear, stress, rearing, behavior

INTRODUCTION

Fear normally functions to protect an animal from danger (1). However, exposure to fear-inducing stimuli is also a potent stressor associated with activation of the hypothalamic–pituitary–adrenocortical (HPA) axis. Therefore, fear may have negative consequences for animal welfare and productivity if the fear response is exaggerated, inappropriate or expressed in a restrictive environment (2–5). Fearfulness is the predisposition of an individual to be easily frightened (1, 6) and is influenced both by genetic and developmental factors.

The early environment may have a large impact on the development of fearfulness and associated activation of the HPA axis in response to stressors (6–9). Exposure to increased environmental complexity during rearing has been found to reduce fearfulness during adulthood in several species including mice (10), pigs (11), and chickens (12). For laying hens, the housing system during rearing is a major source of environmental variability, illustrated by the large difference between cage- and aviary-rearing systems, but few studies have tested for effects of the rearing system on later fearfulness in laying hens. Johnsen et al. (12) compared floor-housed adult birds reared on sand, straw, or wire from 0 to 4 weeks and found that birds reared on wire were most fearful as indicated by longer durations of tonic immobility in response to manual restraint. Anderson and Adams (3) compared cage-housed adult birds reared in a floor or cage system and found that floor-reared birds were more active and displayed more flighty responses to a human than cage-reared birds. A similar study failed to find differences in escape or tonic immobility responses between floor- or cage-reared laying hens housed in cages as adults (13). Other studies testing for effects of exposure to varying degrees of environmental complexity confound effects of rearing and housing of adult birds (14, 15). To the best of our knowledge, there are no previous studies comparing the effects of rearing in a complex aviary system with rearing in a barren cage environment on fear responses in birds housed in the same environment as adults. This knowledge is required for a better understanding of the characteristics of laying hens reared in aviaries or cages under conventional production conditions.

There is a consensus that an individual's fearfulness can be quantified by observing its response to potentially dangerous animate or inanimate objects (6, 16–18). Novel object (NO) tests and human approach tests measure the conflicting motivations to approach and avoid an object as described by Miller's Model (19, 20). According to Miller's Model, an animal will approach an aversive object up to the point at which the motivation to avoid the stimuli becomes as strong as the motivation to approach it (19–22). Fearful animals exposed to potentially dangerous objects typically show escape attempts, avoidance, longer latencies to approach and immobility as well as elevated activation of the HPA axis or sympathetic nervous system, depending on contextual variables and the animals' behavioral strategy (17, 23). However, sometimes it is unclear which variables represent the best measures of fearfulness in a given test situation.

Previous studies also indicate that early experience with a more complex environment may increase the ability of birds to use elevated perches and improve their ability to solve spatial tasks as adults (24–26). This is likely due to effects of sensory stimulation, locomotor experience, and exercise of brain structures underlying cognitive processes as well as neuromuscular systems (26). On this basis, one would expect birds reared in a complex aviary system to use elevated areas of the home cage more often than birds reared in barren cages.

The aim of this study was to test the hypothesis that birds reared in a complex aviary system with exposure to mild intermittent stressors would be less fearful, less sensitive to stress, and use elevated areas of the pen more often as adults than laying hens reared in a simpler cage environment.

MATERIALS AND METHODS

Subjects and Rearing Treatments

The study was conducted using non-beak trimmed, female Dekalb white chickens (*Gallus gallus domesticus*), aged 0–23 weeks with normal health status. Birds were hatched at a commercial hatchery and then reared in separate corridors in a single room until 16 weeks of age. Each corridor in the room contained either a cage- or an aviary-rearing system. The housing system in the single room in which all birds were housed was Natura Primus 1600 (Big Dutchman; http://www.bigdutchmanusa.com) designed for aviary-rearing of laying hen pullets. This system consists of cages stacked in three tiers placed on either side of a corridor for allowing inspection by the caretaker. Cage dimensions are 120 cm × 80 cm × 60 cm (length × width × height). Each aviary cage contains a 120 cm feed trough, one 120 cm perch, and five drinking nipples. All cages can be opened at the front, so that birds can move between each tier and the floor of the corridor. Ramps run from the floor to the second tier to increase ease of access for pullets. When cage doors are in the open position, perches extend from the front of the first and second tiers. The density was 25 birds/m^2 for both treatments during the first 4 weeks of life.

At delivery to the rearing farm immediately following hatching, all chicks were initially placed in cages on the first and second tiers. Chick paper covered 30% of the wire mesh floor of the cages in sufficient amounts to last until the birds were released out in the corridors. At 4 weeks of age, aviary-reared birds (half of the birds in the house) were released from these cages by opening cage doors and allowed to move between the floor of the corridor and each aviary tier on each side of the corridor until the end of the rearing phase at 16 weeks of age. Aviary-reared birds and cage-reared birds were housed in separate corridors throughout the rearing phase. The cage-reared birds (the other half of the birds in the house) were kept inside cages of the first and second tiers until the end of the rearing phase at 16 weeks of age, after which a random subset of birds reared according to each treatment was moved to the experimental facilities.

During rearing, all birds were exposed to the same light intensity, light schedule, and temperatures, as recommended by the General Management Guide for Dekalb White Commercial Layer (27). They were provided with *ad libitum* access to feed using a chain dispersal system and *ad libitum* access to water. The feed type was conventional pullet feed produced and sold by Felleskjøpet, Norway ("Kromat oppdrett 1" for 0- to 6-week-old

birds, "Kromat avl egg 1" for 6- to 8-week-old birds, and "Kromat oppdrett 2" for 8- to 15-week-old birds).

Housing, Feeding, and Lighting at Experimental Facilities

The house was 60 m × 20 m and contained 52,000 chickens in total. At 16 weeks of age, 240 birds from each rearing system (480 birds in total) were transported 490 km by car in transport crates to the experimental poultry facilities at the Norwegian University of Life Sciences, Campus Ås, Norway. At the experimental facilities, they were housed in custom built pens in two adjacent rooms. The two rooms were identical in size and shape and measured 5.90 m × 4.90 m. Each room contained 22 pens. Twenty pens per room contained experimental birds and the remaining two contained reserve birds that were not used in the study. Each room thus contained a total of 240 experimental birds. Each pen's dimensions were 120 cm × 80 cm × 200 cm (length × width × height), and pens were built out of wire mesh on a wooden frame. Each pen contained a wooden nest box (40 cm × 60 cm × 20 cm), an elevated platform (80 cm × 50 cm) at a height of 110 cm, and two perches (80 cm long), one at 70 cm and one at 140 cm above the floor. Each pen contained 12 birds. Birds were housed in mixed groups of six aviary-reared birds and six cage-reared birds per pen (see the Discussion section for a discussion of pros and cons of mixed housing). The experimental pens were numbered 1–20 (room 1) and 21–40 (room 2). On arrival, the birds from both treatments were randomly assigned to a pen. All the birds were fitted with a transparent thin plastic band around the right leg. The end of the plastic band was cut off at 90° (cage-reared birds) or at 45° (aviary-reared birds) to identify the treatment group to which each bird belonged. Also, colored spray paint was used to ease the identification of each treatment group from a distance and thus minimize the handling necessary to collect birds before testing. The birds were sprayed with blue spray paint from wing to wing or with dark green paint from the shoulder blades to the tail. Both markings were allocated to both treatment groups (alternating between pens) to preclude confounding effects of treatment and type of color marking. This identification system was used to ensure that observers were blind to treatment conditions when scoring the distribution of birds in the home pen.

The experimental facility in which adult hens were housed operated on a light cycle that was altered according to recommendations by the Dekalb Management Guide (27). This involved exposure to 100 lux for 24 h after arrival followed by 5–7 lux during the light cycle. Feed was provided *ad libitum* using a circular feeder (50 cm in diameter) hanging 20 cm above ground level. Water was provided *ad libitum* by nipple drinkers (two per pen) mounted 30 cm above ground at the back of the cage. Birds were manually fed with Fjør Oppdrett Lett (Felleskjøpet) until start of lay (16- to 18-week-old birds) and Fjør Egg (Felleskjøpet) until the end of the experiment (24-week-old birds).

Behavioral Tests in the Test Arena

The behavioral tests were performed at 19 weeks ($n = 80$) and 23 weeks of age ($n = 80$). Each bird was only tested once. All birds were tested in a combined voluntary human approach and a NO test. During the test periods, two birds from 10 different pens (five pens per room) were tested each day over a four-day period. From each pen, two aviary-reared birds and two cage-reared birds were tested. The test order of birds was balanced across the room, the distance from pen to the door, and the two rearing treatments. When entering a pen to test a bird, a bird was pseudo-randomly chosen from the floor, the perches, or the elevated platform by the handler. At the first time of testing (19 weeks of age), all birds came from pens with odd numbers. At the second time of testing (23 weeks of age), all birds came from pens with even numbers. The procedure was otherwise the same as for testing at 19 weeks.

The test room measuring 4.90 m × 5.90 m contained a test arena measuring 210 cm × 180 cm × 120 cm (length × width × height) in one corner. Three of the walls were black and opaque, whereas the fourth wall consisted of netting and was, therefore, transparent. The human or the NO was positioned 20 cm outside the netting. When sitting in front of the arena, the stimulus person in the human approach test looked directly toward the arena. The light intensity (measured at chicken height in the test arena) was 7 lux and the sound level between 40 and 60 dB (depending on fan speed). Every morning of the test days, reserve birds from the extra pens that were not used in the experiment were picked up to standardize disturbance of birds before testing. In this way, also the test animals that were tested first had already experienced birds being caught and handled in a different pen prior to testing. The test animals were individually carried on the arm of the worker a distance of 10–20 m from the home pen to the test arena. The time from approaching the bird in the home pen to entering the test arena was 54.7 s (mean) ± 8.24 (SD). The recording of the birds was done by two cameras: one (Panasonic, WV-CP500/G) was suspended from the ceiling, positioned so that it faced the middle of the test arena and connected to a computer with EthoVision XT 10 (Noldus Information Technology, Wageningen, The Netherlands), and the other camera was a camcorder (Canon, Legria HFM56) mounted on a tripod that recorded birds from a position adjacent to the human or NO (**Figure 1**).

A single observer functioned as the stimulus person in all the human approach tests. She was positioned 20 cm outside the netting wall, wore black tights and a blue overall. She sat quietly facing the arena, avoided eye contact with the bird, and tried to keep movements to a minimum during testing. The bird was put into a start box outside one corner of the test arena farthest away from the human, so that it entered into zone 5 (**Figure 1**). The bird was placed into the arena by another experimenter so that by the time the bird entered the arena, the stimulus person was already positioned on the chair. The duration of the test was 5 min. After testing, the bird was left in the arena and exposed to the NO test, as described below.

The stimulus object for scoring the flight response for the NO test was a beige umbrella. The flight response was scored as the umbrella was opened at the beginning of the NO test. As soon as the human approach test was completed, the stimulus person had 5 s to open the umbrella, place it on the chair in front of the arena, and move out of sight of the bird (see **Figure 1**). The duration of the NO test was 5 min. Birds were returned to the home pen directly after the testing.
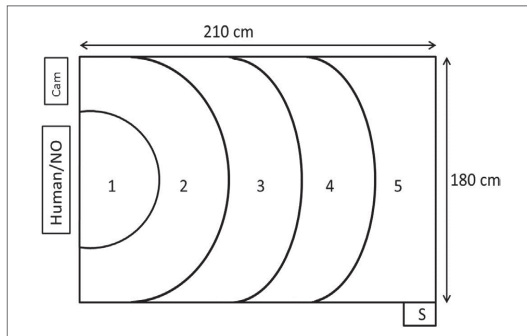
FIGURE 1 | Figure illustrating the test arena where the human approach test and the novel object test was performed. The human or novel object (NO) was positioned just outside the transparent wall. The birds were placed in the arena through the start box (S). The EthoVision XT camera was mounted in the ceiling above zone 3 and pointed down. There was an additional camera (cam) next to the human/novel object. Numbers 1–5 represent zones of increasing distance from the test stimuli.

Behavioral Registrations in the Human Approach and Novel Object Tests

EthoVision XT (Noldus Information Technology, Wageningen, The Netherlands) was the software used to calculate the following variables: distance moved during each test, duration of time spent standing still, and the total time spent in areas of the arena closest to the stimuli (zones 1–4; see **Figure 1**). The birds' response when the umbrella was opened at the beginning of the NO test was categorized as flight or no flight by a blind observer.

Collection and Analysis of Fecal Samples

After the NO test had been completed, the animal was marked with an additional thin, yellow plastic leg-ring to make sure that the same animal would not be tested again. The number of droppings during the 10 min (defecation frequency) in the test arena was recorded, and all feces were collected for analysis of corticosterone metabolites. The fecal samples were stored in a freezer at −80°C until analysis using an enzyme immunoassay (EIA). Droppings were extracted with 60% methanol [0.5 g + 5 ml; (28)] and corticosterone metabolites were measured in an aliquot (after 1:10 dilution in assay buffer) of the supernatant. Measurement was performed with an EIA, which has been successfully validated for non-invasive evaluation of adrenocortical activity in chicken [for details of the assay, see Ref. (29)]. Extraction was performed at NMBU. The EIA was performed at the University of Veterinary Medicine, Vienna.

Behavioral Registrations in the Home Pen

At 19 and 23 weeks of age, the spatial distribution of birds in the home pen was recorded. Observations were done twice daily between 09:30–10:00 and 15:00–16:00 by two observers balanced across the two housing rooms. The observer walked down the aisle of the room, counting the number of birds with each type of spray mark that were (a) perching on the upper perch, (b) sitting on the elevated platform, (c) perching on the low perch, or (d)

on the floor. The number of birds positioned on the floor was calculated by subtracting the birds that were counted from the total number of birds in the pen. The recording took 10–15 min per room.

Ethical Statement

This study was approved by the Institutional Animal Care and Use Committee at the Norwegian University of Life Sciences under ID number 6190.

Data Treatment and Statistical Analysis

The statistical software JMP version 11.0 was used for all statistical analysis (SAS Institute Inc., NC, USA) except where stated otherwise. The pattern of correlation between the continuous test variables (distance moved, duration of standing, and duration of time spent in the four zones closest to the stimuli) in the behavioral tests was analyzed using principal component analysis (PCA) in order to interpret and reduce the number of variables. The variables included in the PCA were total distance moved in the duration of the test, duration of standing still, and the duration of time spent close to the human or NO for both tests, so that the PCA was run on six variables in total. The distribution of variables indicated that no transformations were necessary prior to running PCA. A detailed description of the PCA is provided in Hatcher (30). Principal components were retained for further interpretation if they had an Eigenvalue >1 (the Kaiser criterion), and the scree plot showed a clear separation between retained and unretained principal components and they were interpretable (30). Furthermore, variables were required to have a loading of >0.40 (30). In accordance with a study by Campler et al. (31), no rotation was used. Rotation was not used partly because only one principal component was retained (30), meaning that rotation would be meaningless, and because we were interested in the empirical relationships between variables related to general fearfulness and not in separating these into different stimulus-specific dimensions. The component that was retained was used to generate component scores for individual birds in order to test for treatment effects using ANOVA.

Principal component scores were checked to confirm that they fulfilled the assumptions of general linear models (independence, normality of residuals, homogeneity of variance, and linearity). The ANOVA model was $Y = $ pen' + treatment + pen' × treatment. Because two hens from the same treatment were tested from each pen, we used pen and not hen as the experimental unit to avoid pseudo replication. Pen was a random factor and treatment was a fixed factor.

The flight response when birds were first exposed to the NO was categorized as a nominal variable (flight or no flight). The effect of treatment on whether birds showed a flight response was analyzed using logistic regression in Stata (STATA SE 14.0 for Windows). Analysis was run separately for the two ages (19 and 23 weeks of age). Both treatment (aviary vs. cage) and the zone (1–5) in which the bird was positioned when the umbrella opened were included in the model. For treatment, aviary-reared birds were compared to cage-reared birds, and for zone, zones 1, 2, 3, and 4 were separately compared with zone 5 (start zone

farthest from the NO). The interaction between treatment and position was tested in the model but was not significant and led to a higher Akaike information criterion and Bayesian information criterion and was therefore removed. Very few birds were positioned in zone 1 when exposed to the NO: two aviary-reared birds at 19 weeks of age, both showing a flight response, and one aviary-reared and one cage-reared bird at 23 weeks of age, none of them showing a flight response. Thus, we did not have all combinations of treatment and flight response for zone 1 at any age, and the comparison between birds starting in zones 1 and 5 could not be carried out. In consequence, the four observations from zone 1 were removed from the dataset, giving a total of 78 data points per age. Odds ratios (OR) and p-values are reported. The significance of the whole model was assessed by the likelihood ratio test.

Flight in response to sudden stimulation is sometimes used as an indicator of fearfulness. The relationship between individual scores for the principal component related to fearfulness and the flight response, when exposed to the umbrella, was therefore tested by logistic regression (STATA SE 14.0 for Windows). Flight response was treated as a dependent variable, and the principal component score was used as an independent covariate.

The defecation frequency was analyzed using Fisher's exact test. A total of 148 fecal samples were obtained for analysis of corticosterone metabolites. The corticosterone metabolite data fulfilled the assumptions of General Linear Models and were analyzed using the model described for the principal component scores. The results of the defecation frequency are presented as median, 25th and 75th percentiles, whereas the results for the concentration of corticosterone metabolites are presented as means ± SDs. The data from the home pen observations were treated as follows. The number of hens from each treatment on the top perch, the elevated platform, and the low perch was counted and then divided by the total number of hens from the relevant treatment to give the percentage of birds from each treatment that were found on each of the three different levels. We then calculated the average number of birds over the four days in each position in the pen during the periods of observation. The resulting data were analyzed by Wilcoxon matched-pairs signed-rank sum test, while treating the relative number of aviary-reared birds and the relative number of cage-reared birds in each position as matched pairs. The median, 25th and 75th percentiles, are given for the different locations.

RESULTS

Between the time of delivery at the experimental facilities and the time of study, three animals were excluded due to injuries including two cage-reared birds in two different pens and one aviary-reared bird in a third pen.

Principal Component Analysis and Analysis of Principal Component Score

The PCA generated six components (see **Table 1**). Only the first component (Component 1) fulfilled the criteria for interpretation

(based on Kaiser criterion and scree plot). Component 1 accounted for 55% of the total variation in the data and was highly correlated to all of the test variables. Except for duration of standing still in both tests, all loadings on Component 1 were negative. A high score on Component 1 indicated that a bird spent more time in the area farthest from the NO and human, indicating a high degree of avoidance or a lack of approach. A high score also indicated that a bird spent less time moving and more time standing still. The aviary-reared birds had a lower score for Component 1 compared with the cage-reared birds both at 19 weeks [aviary-reared: -0.2439 ± 1.5560. cage-reared: 0.7437 ± 1.7232. $F_{(1,19)} = 5.6609$; $p = 0.0280$] and at 23 weeks of age [aviary-reared: -0.6864 ± 1.6178. cage-reared: 0.1865 ± 2.1125. $F_{(1,19)} = 4.4907$; $p = 0.0493$; **Figure 2**].

Flight Response

Flight responses are shown in **Figure 3**. For data from birds tested at 19 weeks of age, the model was highly significant (Likelihood ratio test: chi-square $= 13.68$, $p = 0.0084$). Aviary-reared birds tended to have higher odds of showing a flight response than cage-reared birds (OR $= 2.4$, $p = 0.086$). Birds in zones 3 and 4 had higher odds of showing a flight response compared to birds in zone 5 (zone 3: OR $= 7.1$, $p = 0.007$; zone 4: OR $= 4.9$, $p = 0.017$). There was no interaction between treatment and zone.

For birds tested at 23 weeks, the model tended to be significant (Likelihood ratio test: chi-square $= 8.88$, $p = 0.064$). At 23 weeks of age, there was no difference between aviary-reared and cage-reared birds in the probability of showing a flight response (OR for aviary-reared compared to cage-reared birds: 1.4; $p = 0.6$). However, birds in zones 2 and 3 had higher odds of showing a flight response than birds in zone 5 (zone 2: OR $= 5.0$, $p = 0.055$; zone 3: OR $= 7.4$, $p = 0.018$). There was no interaction between treatment and zone.

The OR of flight vs. no flight was not significantly influenced by the principal component score (OR $= 0.89 \pm 0.08$; $z = -1.34$; $p = 0.18$).

Defecation Frequency and Corticosterone Metabolites in Feces

For both treatments in both weeks, the defecation frequencies were low (overall median $= 1$; 25th–75th percentile $= 1$–2), and no significant effects of treatment or week were found (Fisher's exact test; $p > 0.570$). There was no effect of treatment on the concentration of corticosterone metabolites either at 19 weeks (mean ± SD) [aviary-reared birds: 174 ± 45 ng/g; cage-reared birds $= 183 \pm 60$ ng/g; $F_{(1,18.63)} = 0.4728$; $p = 0.5002$] or at 23 weeks of age [aviary-reared birds: 151 ± 67 ng/g; cage-reared birds: 166 ± 70 ng/g; $F_{(1,16.21)} = 1.1421$; $p = 0.3009$].
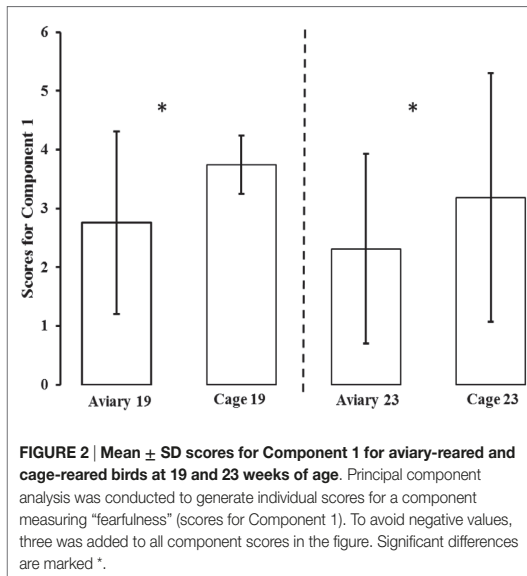
Home Pen Data

At 19 weeks, the aviary-reared birds were observed significantly more often on the top perch, elevated platform, and low perch, compared to the cage-reared birds (**Table 2**). At 23 weeks of age, there was no longer an effect of treatment on the distribution in the home pen.

TABLE 1 | Loading matrix from the principal component analysis (PCA) based on behavioral tests at 19 and 23 weeks of age.

	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6
Human approach test						
Distance moved (cm)	**−0.8908**	−0.2171	0.2030	−0.0276	−0.2885	−0.1846
Duration standing still (s)	**0.7819**	0.2413	−0.3455	**−0.4371**	−0.0718	−0.1217
Duration 1–4 (s)	**−0.6396**	**−0.6356**	−0.1972	−0.3168	0.2164	0.0291
Novel object test						
Distance moved (cm)	**−0.8421**	0.3959	−0.0666	−0.2441	−0.1937	0.1808
Duration standing still (s)	**0.7425**	**−0.5504**	−0.1903	0.0536	−0.3079	0.1088
Duration 1–4 (s)	**−0.5007**	0.0931	**−0.8190**	0.2612	−0.0060	−0.0414
Eigenvalue	3.325	0.9778	0.9108	0.4229	0.2676	0.0960
Variation explained (%)	55.417	16.296	15.179	7.048	4.460	1.600
Cumulative variation (%)	55.417	71.714	86.893	93.940	98.400	100.00

The PCA generated six components (Comp. 1–6). Component loadings >0.40 are written in bold. Duration 1–4 indicates the duration of time spent outside of the area farthest away from the novel object or human.

FIGURE 2 | Mean ± SD scores for Component 1 for aviary-reared and cage-reared birds at 19 and 23 weeks of age. Principal component analysis was conducted to generate individual scores for a component measuring "fearfulness" (scores for Component 1). To avoid negative values, three was added to all component scores in the figure. Significant differences are marked *.

DISCUSSION

Summary

The aim of this study was to test the hypothesis that exposure to increased environmental complexity during rearing reduces fearfulness and increases use of three-dimensional space in adult laying hens. The PCA identified one meaningful component that was used to generate individual scores related to fearfulness, as discussed below. Analysis of treatment effects on scores for this component confirmed that aviary-reared birds housed in the more complex environment were less fearful than cage-reared birds both at 19 and 23 weeks of age. There was a tendency for more aviary-reared birds to fly when startled compared to cage-reared birds at 19, but not at 23 weeks, suggesting that birds reared in the more complex environment initially tend to have a more active behavioral response to the acute fear-inducing stimuli. Observation of the birds' behavior in their home pens indicated

a transitory effect of rearing in which aviary-reared birds more often used the elevated parts of the pen than cage-reared birds at 19, but not at 23 weeks of age, suggesting that rearing in a more complex environment increases three-dimensional spatial orientation or motor skills. The rearing treatment had no effect on defecation frequency during behavioral testing or on the concentration of fecal corticosterone metabolites at either age. The latter finding suggests that there were no treatment effects on basal HPA-axis activity.

Principal Component Analysis

Fearfulness is the predisposition to avoid different potentially dangerous stimuli as measured using the duration of time spent farthest away from the NO or human in our behavioral tests. Therefore, we interpret Component 1 as reflecting fearfulness. Other variables loading on Component 1 indicated that more fearful birds moved less and spent more time standing still. This corresponds well to interpretations of behavioral inhibition and lack of locomotion as a frequently used indicator of fearfulness in laying hens (17). Our interpretation also corresponds well with a similar study indicating that standing or sitting alert and locomotion recorded in some fear-inducing situations in laying hens were related to the same principal component (31). The remaining components in the current study were related to such few variables that interpretation would be highly speculative.

Treatment Effects on Fearfulness

Analysis of treatment effects on scores for Component 1 interpreted as fearfulness as discussed above, confirmed that aviary-reared birds housed in the more complex environment were less fearful than cage-reared birds at both ages. This corresponds well to findings by Brantsæter et al. (32) in which cage-reared birds were more hesitant than aviary-reared birds to approach a NO in their home cage. The current study used PCA analysis to generate a fearfulness score that took account of six variables across two different test conditions in which birds were exposed to a variety of stimuli. The PCA score used in the present study may be a better measure of fearfulness than the single response variable used by Brantsæter et al. (32), as the latter may be more stimulus specific.

FIGURE 3 | Figures showing the flight response for aviary-reared and cage-reared birds at 19 and 23 weeks of age. Black columns indicate aviary-reared birds. White columns indicate cage-reared birds. The *x*-axis indicates the zone. Zone 1 was closest to novel object and zone 5 was the start zone farthest away from the novel object. The *y*-axis indicates the number of birds that did not fly at 19 weeks of age **(A)**, the number of birds that flew at 19 weeks of age **(B)**, the number of birds that did not fly at 23 weeks of age **(C)** and the number of birds that flew at 23 weeks of age **(D)**.

TABLE 2 | Showing results of the Wilcoxon test for distribution of birds in the home pens at 19 and 23 weeks of age.

		Aviary-reared birds		Cage-reared birds		Test statistic *S*	*p*-value
		Median	25th–75th percentile	Median	25th–75th percentile		
19 weeks	Top perch	16.67	10.42–22.92	5.21	2.08–14.06	−289	<0.0001
	Elevated platform	19.20	13.02–25	12.08	6.25–22.08	−183	0.0088
	Low perch	20.83	14.36–22.92	12.5	10.42–18.44	−204.5	0.0045
23 weeks	Top perch	9.38	4.69–14.58	6.25	2.08–10.42	−41	0.1014
	Elevated platform	12.5	7.71–22.40	6.25	4.17–16.15	−34.5	0.1381
	Low perch	15.63	10.94–18.75	13.54	8.33–18.75	−30	0.2364

At 19 weeks, aviary-reared birds were positioned significantly more often on all elevated areas in the home pen, whereas at 23 weeks of age there was no difference between the treatment groups.

Treatment Effects on Flight Response

Aviary-reared birds tended to fly more when the umbrella was opened than the cage-reared birds at 19 weeks, but not at 23 weeks of age. The flight response provides information about how actively the birds responded when exposed to unexpected, abrupt event. The flight response is similar to responses observed in flocks of birds living under production conditions that respond to sudden exposure to novel stimuli. Such panic responses may result in clumping and mortality by suffocation of birds located at the bottom of heaps that might form. Therefore, the tendency for aviary-reared birds to be more predisposed to fly in response to sudden exposure to novelty suggests that they might have more trouble with clumping in loose housing systems than cage-reared birds. However, this disadvantage must be weighed against the many disadvantages of housing cage-reared birds in aviaries regarding problems with navigation (26) and use of perches and nest boxes (24, 25). Some authors interpret flight responses as an indication of elevated fearfulness (3, 33). However, this interpretation is

questionable in light of the findings in the current study showing a lack of any relationship between fearfulness as indicated by principal component scores and flight response. We propose that flight in response to acute exposure to novel stimuli in laying hens rather reflects the coping style of birds. This interpretation would suggest that rearing in a more complex and challenging environment tends to make birds more proactive (34).

An aspect of our experimental design that may have influenced the rearing effect on flight is the position of the bird in the arena at the time the umbrella was opened. Aviary-reared birds, which came closer to the human during the voluntary human approach test, might have been more intensely stimulated than birds that were positioned further away. If this is correct, it means that aviary-reared birds would have been exposed to a higher degree of stimulation when the umbrella was opened. This may have increased the likelihood of flying in this treatment group. However, the lack of interaction between treatment and zone indicated that this was not the case.

Defecation and Corticosterone Metabolites

To the authors' knowledge, no previous studies have compared defecation frequency in birds subjected to rearing conditions with different degrees of environmental complexity. In rodents, defecation frequency is widely used to assess the stress levels experienced by the animal in behavioral tests (1, 35–38). In chickens, defecation frequency is not as common to measure but is sometimes reported as a measure of underlying fearfulness (39–42). The present study did not detect an effect of environmental complexity during rearing on defecation frequency. The concentration of corticosterone metabolites in the feces is considered as an indirect measure of the level of circulating plasma corticosterone. Measuring fecal corticosterone metabolites is an increasingly used method for non-invasive quantification of chronic stress (43–45). Plasma corticosterone is mainly metabolized by the liver and can be found in the feces approximately 4 h after an induced increase in blood levels (46). Therefore, a treatment effect on corticosterone metabolite excretion would indicate higher basal activity in the HPA axis, but this was not found in the current study. Future studies could include tests for rearing effects on the HPA response to acute stress using analysis of blood samples.

Space Use in the Home Pen

At 19 weeks of age, most of the cage-reared birds were observed on the floor of the home pens, whereas more aviary-reared birds were positioned on the perches or the elevated platform. At 23 weeks of age, this treatment effect had disappeared. The effect at 19 weeks of age suggests that aviary-reared birds are more aware of perches and elevated areas of the pen or that they have better-developed motor systems. This finding and interpretation corresponds to previous studies (24–26). This temporal development in treatment effects on the use of elevated areas of the home pens corresponds well to the treatment effects on the flight response as previously discussed.

Pros and Cons of Housing Both Treatments in the Same Pens

In this study, we cohoused birds from both treatments. This was considered necessary to increase power and exclude the possibility of confounding effects of pen and treatment. However, fearful individuals can influence their conspecifics (47, 48). At the most extreme, this transmission can cause whole flocks to panic (2). By housing the treatment groups in mixed pens, the birds could influence each other and become more similar over time. In the present context, this would be a conservative source

of error, tending to reduce the likelihood of finding treatment effects.

Animal Welfare Implications

The current study was conducted when the birds were between 19 and 23 weeks of age. At 19 weeks of age, birds have been transported from the rearing farm and are starting to lay. This is therefore a time in the life of laying hens at which their ability to cope with fear-inducing environmental changes and challenges may be especially important for their welfare and productivity. The present findings, therefore, suggest that laying hens reared in a more complex system are better equipped to cope with the challenges to which they are exposed to around the onset of lay.

CONCLUSION

This study confirmed our hypothesis that environmental complexity during rearing has an effect on the development of fearfulness in laying hens. The fear tests conducted at 19 and 23 weeks of age revealed that aviary-reared birds were less fearful compared to the cage-reared birds. The rearing treatment did not affect defecation frequency during testing or basal corticosterone metabolite concentrations. The latter suggests that varying environmental complexity does not influence basal activity in the HPA axis.

AUTHOR CONTRIBUTIONS

MB collected the data, analyzed the data, did practical work at the study site, and wrote the first draft of the article; JN did practical work at the study site and collected and analyzed the data; AP did practical work at the study site and collected data; TR contributed to conception of the study; AJ led the project, participated in conception and design of the study, and did practical work at the study site. All authors contributed to planning the study, writing the manuscript, and approved the final version.

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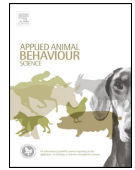
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Paper III

Access to litter during rearing and environmental enrichment during production reduce fearfulness in adult laying hens

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ABSTRACT

Exaggerated fear-reactions are associated with injurious flying, smothering, feather pecking and other events that compromise animal welfare in laying hens. The aim of this study was to test the hypothesis that chicks with access to litter during the first five weeks of life would be less fearful as adult hens compared to birds reared without access to litter. The hypothesis was tested in a national on-farm study in commercial aviary flocks in Norway. Five rearing farmers divided the pullets into two groups within their rearing houses. While the chicks were enclosed inside the aviary rows during the first five weeks of life, paper substrate where food and other particles could accumulate, covered the wire mesh floor in the treatment group, whereas the control group was reared on bare wire mesh. At 30 weeks of age, 23 aviary flocks (11 control flocks reared without paper and 12 treatment flocks reared with paper) were visited. During the visit, the fearfulness of the adult birds was tested in a stationary person test and a novel object test. The data was analysed by ANOVA or logistic regression as appropriate. The access to litter during rearing did not influence the number of birds that approached within 25 cm of the stationary person ($p = 0.51$). All flocks, regardless of rearing treatment, had birds which came within 2 m of the stationary person. The latency to approach within 2 m of the stationary person tended to be influenced by provision of environmental enrichment as adults ($p = 0.08$) and by the interaction between treatment × rearing farm ($p = 0.08$). The number of birds that approached within 2 m of the stationary person was influenced by the interaction between treatment during rearing and provision of enrichment as adults ($p = 0.03$), however, the *post hoc* test showed no pairwise differences. All flocks, regardless of rearing treatment, had birds that approached the novel object. The access to litter during rearing did not influence the birds' latency to approach the novel object. The number of birds approaching the novel object was affected by the interaction between access to substrate during rearing and provision of environmental enrichment as adults ($p = 0.05$). The results indicate that both adding paper substrate to chicks from the first day of life and environmental enrichment as adults, reduce fearfulness in laying hens.

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1. Introduction

Following the EU ban on conventional cages (99/74/EC), laying hens are either housed in enriched cages or loose housed (barn or aviary systems). The law requires that all adult hens have unrestricted access to a feed trough, nest boxes, perching space and litter which allows pecking and scratching (Comission, 1999). Several countries including Norway, Sweden, Germany, Austria and The Netherlands mainly keep laying hens in loose housing systems (floor housing or aviaries) (Comission, 1999; Erhvervsfjerkræsektionen, 2015). The change from conventional cages was intended to improve animal welfare, as loose housing systems enable the birds to express a greater variety of highly motivated natural behaviours such as perching, dust bathing, foraging

and laying in a nest box (Lay et al., 2011; Cronin et al., 2012; Janczak and Riber, 2015). However, loose-housed birds do not necessarily experience increased welfare, as indicated by higher mortality rates and higher risk of developing feather pecking and cannibalism compared to birds housed in cages (Michel and Huonnic, 2003; Tauson, 2005; Rodenburg et al., 2008b; Lay et al., 2011).

Fearfulness is the predisposition of an individual to be easily frightened (Boissy, 1995; Jones, 1996). Under natural conditions, fear functions to protect the animal from danger (Boissy, 1995). However, when the fear response is exaggerated or inappropriate (Mills and Faure, 1990; Jones, 1996) and the environment does not allow for successful coping with the fear inducing stimulus, it may severely compromise the welfare of the animal. For example, fearfulness has been linked to feather pecking (Vestergaard et al., 1993; Jones et al., 1995; El-Lethey et al., 2001; Rodenburg et al., 2004; Rodenburg et al., 2009; Uitdehaag et al., 2009; de Haas et al., 2014a), increased risk of injuries such as keel bone fractures (Harlander et al., 2015) and increased risk of smothering (Hansen, 1976; Mills and Faure, 1990; Bright and Johnson, 2011; Gilani et al., 2012; Richards et al., 2012). Fearfulness has thus important welfare implications for laying hens.

The rearing period affects development and is thus crucial in preparing the birds for the challenges they will encounter during adulthood (Rogers, 1995; Rodenburg et al., 2008a; Janczak and Riber, 2015). The early environment influences the development of fearfulness and associated activation of the hypothalamic–pituitary–adrenocortical axis in response to stressors (Jones, 1996; Caldji et al., 2000). Some rearing-associated factors found to influence fearfulness in poultry are environmental complexity (Jones, 1982; Reed et al., 1993; Brantsæter et al., 2016a, 2016b), group size (Bilcik et al., 1998; Rodenburg and Koene, 2007), and access to brooders (Gilani et al., 2012; Riber and Guzman, 2016), perches (Brake et al., 1994), or outdoor areas (Grigor et al., 1995; Tobias Krause et al., 2006). Exposure to the aforementioned factors is mainly dictated by the design of the physical environment and optimising economical profit, whereas management-related rearing factors are more malleable. Birds exposed to more human contact during rearing have been reported to have lower levels of fearfulness (Jones, 1993; Reed et al., 1993; Zulkifli, 2008; Edwards et al., 2010). Another more practical, albeit controversial, management-related procedure is early provision of pecking substrate (litter) to the pullets during the time before release from aviary rows (Aerni et al., 2005; de Haas et al., 2014a; de Haas et al., 2014b). However, differing opinions regarding provision of substrate are based on practical experience and anecdotal evidence rather than systematic investigation.

Previous work (de Haas et al., 2014b) indicated that chicks experiencing disruption or limitation of litter supply tended to keep a larger distance to a human and to have an increased latency to approach a novel object, compared to birds reared with constant access to litter during early rearing. This was probably caused by the fact that the treatment caused an increase in severe feather pecking and feather damage, already at a young age, making the birds more fearful. However, all chicks were originally given access to chick paper, so the effect of having no litter from the very beginning was not investigated (de Haas et al., 2014b). Furthermore, the birds in that study were beak trimmed which can influence the results (Davis et al., 2004; Janczak and Riber, 2015). Another study comparing birds reared on wire, straw or a combination of sand and straw, found that the birds reared without access to litter had longer durations of tonic immobility, an indicator of fearfulness (Jones, 1986; Johnsen et al., 1998). Early access to litter has also been reported to positively influence egg weight, egg mass and feed conversion ratio and to reduce mortality (Aerni et al., 2005) and feather pecking (Huber-Eicher and Sebö, 2001; Nicol et al., 2001; de Haas et al., 2014a, 2014b; Tahamtani et al., 2016). However, to the authors'

knowledge, no previous study has investigated the effect of access to litter during rearing on fearfulness in commercial adult Lohman selected Leghorns with intact beaks.

The aim of this study was to test the hypothesis that hens reared with early access to pecking substrate would be less fearful as adults compared to hens reared without pecking substrate. We predicted that access to litter in the form of paper substrate in the early days of rearing would result in decreased fearfulness in adult laying hens.

2. Materials and methods

We designed a study following the guidelines for a randomised, blinded, controlled clinical trial (O'Connor et al., 2010). We recruited rearing farmers from across Norway and instructed them to provide part of the animals in the same rearing house with paper substrate from one day of age while the other animals received no paper substrate during the early rearing period. All animals were visited at the production farm at around 30 weeks of age and the flocks were tested for their level of fearfulness.

2.1. Population and treatment allocation

Non beak-trimmed, female Lohmann Selected-Leghorn (LSL-Classic) chickens (*Gallus gallus domesticus*) of up to 32 weeks of age and normal health status were used in this study. In total, 12 rearing farms were assessed for eligibility. Of these, five had the appropriate facilities for the study design and agreed to be enrolled into the study. Among the five rearing farms included, approximately 489,000 laying hens were randomly allocated to one of the two treatment groups. At one day of age, more than 15,000 chicks arrived at each rearing farm and were distributed in the aviary rows of the rearing house. 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 (Fig. 1). In one of these groups, the rearing farmers supplied chick paper (Tork, SCA, The Netherlands) approximate thickness 41 g/m^2 over the wire mesh floor inside the aviary rows. The chick paper prevents the legs of young animals from falling through the wire mesh. It also allows the aggregation of particles such as dust, spilled food and droppings, thus providing the chicks with foraging substrate inside the aviary row from the first day of age. The paper was present from the time the chicks arrived and remained until the birds were released into the corridors of the aviary. 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 wire mesh 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 groups and the animals were allowed to move freely within each corridor containing birds of the same treatment. Some rearing farmers distributed sparse amounts of saw dust before releasing the birds, whereas the majority of rearing farmers relied on the build-up of dust and other particles as "litter" on the floor of the corridors. Due to the physical separation of the aviary rows and corridors, the animals from one treatment group did not mix with animals from the other group. Rearing farmers were asked to repeat the experiment with a second batch of chicks, in the same house, after the first batch was old enough to be transported to production farms. During the second round of experiments, treatment and control rows were reversed in relation to the first round to preclude confounding effects of rows/locations within the rearing house (Fig. 1). All other husbandry procedures, both at rearing farms and at production farms followed recommendations from the Lohmann management guide. At 16 weeks of age, the hens were transported by truck from
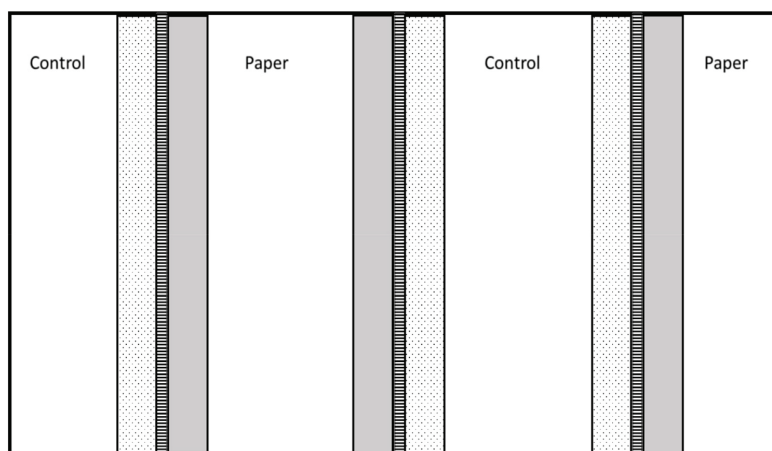
Fig. 1. Schematic diagram of a rearing farm with three aviary rows and four corridors. The pullets were enclosed within the aviary rows, where they have access to food and water, for the first five weeks either on paper (grey areas) or on bare wire mesh (dotted areas). The horizontal lines represent the physical barriers between paper and control group corridors. During the second round of experiments, the control and paper rows were reversed in relation to the first round to preclude confounding effects of rows/location within the rearing house. Each corridor contained at least 7500 chickens.

the rearing farms to the production farms, where they were visited by the researchers for data collection.

2.2. Housing and general procedures

All birds were hatched at a commercial hatchery and then transported at one day of age to five rearing farms in Norway, all of which used aviary rearing-systems (Natura, Big Dutchman, Germany). Upon arrival at the rearing farms, the temperature inside the rearing houses was set to 35–36 °C with a humidity of 60 − 70% for the first two days. The temperature was then gradually decreased to approximately 19 °C at five weeks of age, at which point it was held constant until transport to production farms at 16 weeks of age. The birds were kept in a 4-h light/2-h dark light cycle at 20–40 lx for the first seven days after arrival at the rearing farms. After seven days, the light regime was adjusted to a 14-h light/10-h dark cycle. Subsequently, at each week one hour of light was removed from the light regime until eight hours of light was achieved at eight weeks of age. At four weeks of age, the light intensity was reduced to approximately five lux until transfer to the production farm. Birds were provided with ad libitum access to feed and water during rearing and production. Feed was provided via a chain dispersal system, and water via drinking nipples. All rearing and production farms provided commercial feed for layers with optimised nutritional content for specific ages in accordance with recommendations from the Lohmann Management Guide (Lohmann, 2014).

2.3. Data collection

The birds were only visited by researchers during the laying period. In total, 40 production farms were visited. Each farm contained on average 7500 laying hens from the same rearing farmer 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, at the peak of lay. This time point was chosen as an age where birds are settled into the production environment and the laying percentage is at its peak. Both producers and researchers were blind to which experimental treatment the visited flock belonged. During this visit, which lasted two to three hours, the hens were subjected to two different fear tests: a stationary person test and a novel object test as described below. Only

the researchers were present inside of the house during data collection. All visits were conducted during the light hours of the light cycle, always after the majority of the hens had laid their eggs for the day. The researchers walked in separate parts of the house at all times to ensure minimal disturbance of the hens. Normal routines of the system (e.g. feeder chains, light intensity) were not altered during the assessment. Only one flock was visited per day.

2.3.1. Measuring fearfulness: stationary person test and novel object test

Fearfulness was assessed by a stationary person test and a novel object test. In order to generate a representative average for the flock, the fear tests where each performed at six locations in the chicken house. The tests were thus carried out in all corridors, and at different distances to the door (Fig. 2). Depending on the design of the individual production aviary, the fearfulness tests were performed in the litter area and/or inside the corridors. At each location, the stationary person test was always conducted prior to the novel object test. This ensured that the birds did not habituate to the presence of the observer before the stationary person test was conducted. Also, after the stationary person test, the birds were likely to have accustomed to the observer, and the fearfulness would be a measure of the novel object rather than confounded with fear of the observer. Measures from the six locations were averaged per flock. The response variables generated from the fear tests are presented in Table 1.

The stationary person test was based on methods described by the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009), and papers evaluating fear-tests in loose housing systems (Raubek et al., 2007; Graml et al., 2008a; Graml et al., 2008b; de Haas et al., 2014a; de Haas et al., 2014b). The experimenter walked slowly down the corridor (maximum one step per second) and stopped at six areas distributed throughout the house. At the given location, the experimenter would stand still for a total of two minutes. Every 10 s, aided by a stopwatch, the experimenter counted the number of birds within 25 cm of the experimenter's feet. After the first three farm visits, the protocol was amended to include an additional measure of number of birds within 2 m of the experimenter's feet. At the same location, after the completion of the stationary person test, the experimenter proceeded with the novel object test.
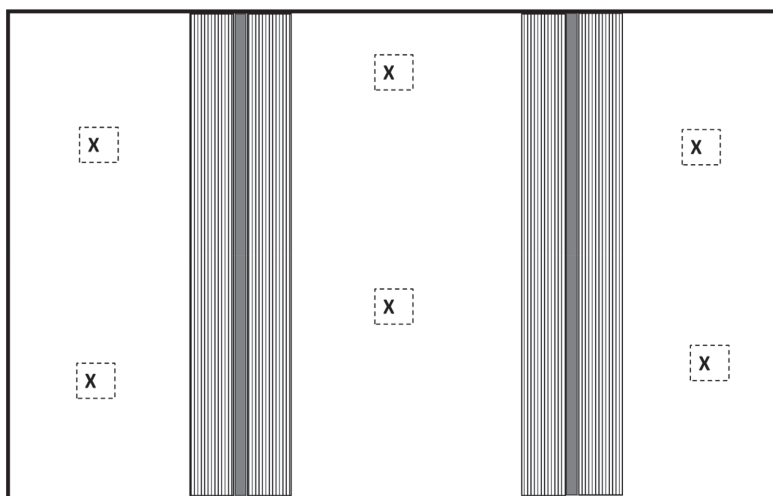
Fig. 2. Schematic diagram of a production farm where the fear tests were conducted when the birds were 30 weeks of age. The grey areas represent the aviary rows where food, water, perches and nest boxes were found. In some production farms, the birds could move across the house from one corridor to the others, whereas in other farms the birds were restricted to one corridor only. The spots marked "X" are examples of where the experimenters would conduct the stationary person test and the novel object tests at six locations of the house to represent the flock as a whole.

Table 1
Response variables, the definitions of the response variables, transformations used, and the statistical test used for each variable. NOT = Novel object test, SPT = stationary person test.

Response variables	Description	Statistical method (transformation in brackets)
NOT approach	Whether one or more hens came within 25 cm of the novel object (yes/no)	Descriptive
NOT latency	The latency for three hens to come within 25 cm of the novel object. Maximum latency 120 s	ANOVA (LOG-transformation)
NOT average	The average number of birds in the flock that approached within 25 cm of the novel object	ANOVA (untransformed)
SPT 2 m approach	Whether one or more birds came within 2 m of the stationary person (yes/no)	Descriptive
SPT 2 m latency	The latency for one or more hens to approach within 2 m of the stationary person. Maximal latency 120 s	ANOVA (LOG-transformation)
SPT 2 m average	The average number of birds in the flock that approached within 2 m of the stationary person	ANOVA (untransformed)
SPT 25 cm approach	Did birds in the flock approach within 25 cm of the stationary person (yes/no)?	Logistic regression

The novel object test was based on the Welfare Quality® Assessment protocol for poultry (Welfare Quality, 2009) and previous protocols conducted on loose housed hens (de Haas et al., 2014a; de Haas et al., 2014b). The novel object used was a 50 cm long stick with different coloured tapes of 3 cm width. As the novel object test was conducted immediately after the stationary person test, the birds had likely accustomed to the experimenter. The novel object was placed on the floor of the corridor not too far from a light source and the experimenter stepped slowly backwards approximately 1.5 m. After placement, every 10 s, the experimenter counted the number of hens within 25 cm (bird length) of the novel object. The test lasted a total of two minutes, the maximum latency to approach the novel object was thus 120 s.

2.3.2. Environmental enrichment

During the visit at the production farms the researchers made notes on the use of environmental enrichment. Environmental enrichment was defined as any supplement in addition to food or water, which encouraged active and explorative behaviour (Newberry, 1995; Jones, 2004). Examples of environmental enrichment applied by production farmers were empty plastic boxes, box lids, toy balls, old CDs, Siporex (aerated concrete and calcium sil-icate hydrate block), saw dust, oyster shell and cut up pieces of manure belt or egg belts. The use of environmental enrichment by the production farms was not regulated by the experiment. Each farm used the type and amount of enrichment that was already part of their management routines. The use of environmental enrichment was categorized by the researchers as "yes" or "no".

2.3.3. Other parameters

During the visit at the production farm, the researchers also made notes on the availability of floor space, ease of hen movement within the house, the age of the flock, and the time of the day the assessment was carried out.

2.4. Inclusion criteria

Only rearing farmers who delivered birds from both rearing groups (with and without paper) were included in the study. The number of treatment and control flocks provided by each rearing farm, depended on the size of the rearing flock. For example, some rearing flocks were large enough to provide multiple production farms. At minimum, each rearing farm provided birds to two production farms, one treatment flock and one control flock. If rearing

Table 2
An overview of the number of flocks provided by each rearing farm, per treatment group.

Treatment	Rearing farms					Total
	1	2	3	4	5	
Control	1	3	2	4	1	11
Paper	3	2	2	4	1	12

farmers were unsure about which treatment the birds originated from, data for the flock were excluded from the final data set. Each production farm received hens from only one rearing farmer and from only one treatment group. Any production farms that received mixed flocks were excluded from the study. Production farms were also excluded from the study if they reported having red mite infestations or had issues with the feed that resulted in a drop in egg laying percentage. Only aviary production farms were visited for the purpose of this study. In addition, the production farms were only included in the study if the aviary system was used appropriately. For example, producers who enclosed the birds within the aviary row for any period of the day or night were excluded from the final dataset.

2.5. Data analysis and sample size

A short description of the response variables and any transformations used are presented in Table 1. Models for Analysis of Variance (ANOVA) were selected by backward and forward stepwise selection. Models with the highest R^2 adjusted were selected for data analysis. All factors included in the final models had p-values <0.1. The variables that were recorded during the visit at the production farm and that were possible to include as factors in the models were treatment (access to paper versus control), rearing farm, whether the birds had access to environmental enrichment at the production farm and age of the flock at the time of the visit. All explanatory variables were fixed. The assumptions of ANOVA (normality of residuals, homogeneity of variance and linearity) were checked, and the variables were transformed if necessary. *Post hoc* testing was performed with Tukey's test (Tukey's HSD test). Presented values are untransformed means ± standard deviations for results analysed by ANOVA. Whether the birds approached within 25 cm of the stationary person, was analysed by logistic regression. The final model included the fixed factors treatment and enrichment. The statistical software used was Jmp version 11 (SAS Institute Inc., NC, USA) for ANOVA, and Stata SE 14 (StataCorp LP) for logistic regression.

In total, five rearing farms were included in the study. They generated 23 flocks of laying hens that conformed to the inclusion criteria, 11 flocks reared without access to paper (control), and 12 flocks reared with access to paper substrate (treatment group) during the first weeks of life (Table 2). Measures from the novel object test and the stationary person test 25 cm were obtained at all 23 farms, whereas the stationary person test 2 m measure was obtained at 20 farms. Out of the 23 production farms included in the dataset, 14 producers provided the birds with environmental enrichment.

3. Results

3.1. Stationary person test

Ten of the 23 flocks had birds that came within 25 cm of the stationary person. No effect of treatment was detected when analysed by logistic regression ($p = 0.51$). The stationary person test for birds within 2 m was conducted at 20 farms. All 20 flocks, regard-
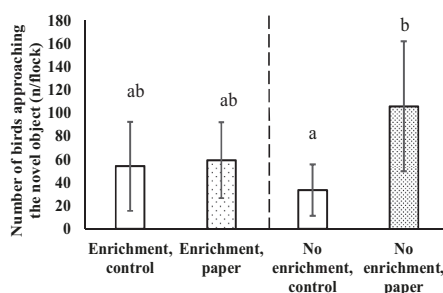
Fig. 3. Each bar represents the mean number (and standard deviation) of birds that approached the novel object. The bars display flocks from the control and paper rearing groups, and whether the birds were provided environmental enrichment as adults. Bars with different letters are significantly different (p < 0.05).

less of rearing treatment, had birds which came within 2 m of the stationary person.

None of the 20 flocks had the maximum latency to come within 2 m of the stationary person, so the variable was analysed by ANOVA rather than survival analysis. The best model to explain the variation in the latency data included treatment + rearing farm + enrichment + (treatment x rearing farm) (R^2 adjusted = 0.25) after LOG-transformation of the latency data. Enrichment ($p = 0.08$) and the interaction between treatment x rearing farm ($p = 0.08$) tended to influence the latency to come within 2 m of the stationary person.

For the number of birds that came within 2 m of the stationary person, the best model to explain the variation in the data included treatment + rearing farm + enrichment + (treatment × enrichment) (R^2 adjusted = 0.13). There was a significant interaction effect of treatment × enrichment ($p = 0.03$), however, *post hoc* Tukey test showed no pairwise differences.

3.2. Novel object test

Birds in all 23 flocks approached the novel object, regardless of treatment group.

As only two farms had the maximal latency to approach the novel object, the variable was analysed by ANOVA rather than survival analysis. The best model to explain the variation in latency included treatment + rearing farm + enrichment + (treatment × rearing farm) (R^2 adjusted = 0.19) after LOG-transformation. The interaction term was not significant ($p = 0.14$), and after removing the interaction term, none of the factors had p-values <0.1.

The best model to explain the variation in data for number of birds approaching the novel object included treatment + enrichment + (treatment × enrichment) (R^2 adjusted = 0.21). There was a significant effect of the interaction treatment x enrichment ($F_{1,19} = 4.35$, $p = 0.05$). The interaction effect indicated that if birds did not have access to enrichment during production, significantly more birds reared with paper approached the novel object compared with birds reared without paper (*post hoc* Tukey $p = 0.04$). For birds with access to enrichment during production, the rearing seemed to have less effect on the number of birds that approached (*post hoc* Tukey $p = 0.99$). There was no difference between enrichment and no-enrichment at the production farm for either the control group (*post hoc* Tukey $p = 0.80$) or the treatment group (*post hoc* Tukey $p = 0.21$) (Fig. 3).

4. Discussion

The present study indicates that providing paper substrate during early rearing reduces fearfulness at 30 weeks of age in laying hens. However, the effect on fearfulness at 30 weeks of age was dependent on whether the birds had access to environmental enrichment as adults. This emphasises the importance and potential welfare implications of management both during rearing and production.

Rearing farmers tend to use personal experience and anecdotal evidence rather than scientific evidence when considering whether to provide substrate to young pullets. Some rearing farmers argue that providing chicks with paper substrate ensures survival of the youngest birds, as they otherwise struggle getting access to food and water, and can get stuck in the wire mesh (N. Steinsland, *personal communication,* September 2015). Other rearing farmers disagree, and do not provide their pullets with litter as they claim the paper clogs the manure belts. The decision regarding substrate provision is thus more focused around practicalities rather than the potential welfare benefits. Although few previous studies have tested the effects of access to substrate during rearing on adult fearfulness, the authors' are aware of one experimental study and one study conducted under commercial conditions that are relevant for comparison (Johnsen et al., 1998; de Haas et al., 2014b). Although the reported effect of substrate provision during early rearing in our study was only evident for birds without access to environmental enrichment as adults, our findings correspond well with previous studies suggesting that provision of litter during rearing reduces fearfulness in laying hens (Johnsen et al., 1998; de Haas et al., 2014b). The study conducted under commercial farming conditions report that beak-trimmed pullets experiencing litter disruption or litter limitation were more fearful compared to birds with constant access to litter, when tested at 10 weeks of age (de Haas et al., 2014b). Additionally, a follow up study of the same birds reported that fear of humans (both during rearing and in the laying phase) was a predictor of feather damage at 40 weeks of age (de Haas et al., 2014a). Likewise, an experimental study comparing the effects of rearing pullets with access to either sand, straw or bare wire mesh the first four weeks of life, indicated that wire-reared birds had longer durations of tonic immobility at 42 weeks of age (Johnsen et al., 1998) than birds reared on the other substrates. However, the results from the study by Johnsen et al. (Johnsen et al., 1998) are not necessarily relevant for commercial settings as the experimental environment and management the birds experienced, both of which are crucial for the outcome of the tests, differ (Dawkins, 2012; Gilani et al., 2012). Our study thus helps fill a knowledge gap on how access to litter affects laying hens under commercial farming conditions.

A common method of operationally measuring underlying fearfulness is to record the responses of birds exposed to novelty. Novel object tests and human approach tests are well validated (Forkman et al., 2007), and measure the conflicting motivations to approach and avoid potentially dangerous stimuli as described by Miller's Model (Miller, 1944, 1959). Our results show an interaction between access to paper during rearing and later access to environmental enrichment both in the number of birds that came within 2 m of the stationary person and for the number of birds approaching the novel object. For the novel object test, this meant that if the birds had access to environmental enrichment after transfer to the laying farm, access to litter during rearing did not affect the number of birds that approached the novel object. On the contrary, for adult birds housed without access to environmental enrichment, more of the birds reared with access to paper approached the novel object compared to birds reared without access to paper substrate. One possible interpretation of this interaction, is that enrichment given to adult hens counteracts the negative effects of not having

access to paper early in life. The activity level of the birds during exposure to fear-inducing stimuli is also affected by the underlying fearfulness, as fearful birds are generally less active compared to birds with lower levels of fearfulness (Brantsæter et al., 2016a). This means that the early exposure to substrate makes birds more prone to approach a novel object and suggests a lower level of underlying fearfulness. As the behaviour recorded in the adult birds was dependant on both litter access as pullets and provision of environmental enrichment as adults, our results correspond with other work in poultry emphasising that behaviour can be altered also during adulthood (Jones, 1982; Reed et al., 1993; Nicol et al., 2001).

In comparison to the effects found in the novel object test, the results from the stationary person test were more difficult to interpret. None of the factors that were included in our statistical models influenced the number of birds that came within 25 cm of the stationary person. The latency to approach within 2 m of the stationary person tended to be influenced by enrichment and by a treatment × rearing farm interaction. For the number of birds that came within 2 m of the stationary person, the *post hoc* test indicated no pairwise differences despite the interaction between rearing treatment and provision of environmental enrichment during production indicated by the main test. In our study, the novel object test was therefore possibly a better measure of fearfulness compared to the stationary person test.

Contradictory to our predictions, the stationary person test did not detect any difference in fearfulness between the treatment groups. A possible explanation of the discrepancy between results in the novel object test and the stationary person test could be that the birds had negative experience with humans during handling or transportation from the rearing farm to the production farm. The novel object itself was new and without any previous negative associations. Another possible reason for the discrepancy between tests could be other sources of variability that we could not control for in the study, such as the width of the corridors where the tests were performed, the light intensity inside the house, and the presence of barriers that could influence the ease with which the birds could approach or avoid the human. For example, in houses with higher light intensity, flocks would see the human more clearly which possibly influenced their approach or avoidance behaviour. On the contrary, the novel object was always placed close to a light source on the ground and the test was therefore less reliant on the light intensity of the house. When following the Welfare Quality® Protocol for poultry (Welfare Quality, 2009), very few flocks came within the suggested distance of 25 cm of the human during testing. When increasing the distance to include a 2 m radius, all flocks regardless of rearing treatment, had birds that entered this area. As the rearing affected responses to the novel object but not the human, it is possible that the latter is related to a lack of sensitivity of the stationary person test. For future studies, we therefore advise making amendments to the protocol to adjust it to the housing system in which birds are studied. One suggestion would be to calculate the exact minimal distance between the birds and the human. Another could be to correct measurements for the ease with which birds can avoid the experimenter (presence of barriers) and for light intensity. For example, in some houses the birds had plenty of space and avoided the experimenters, not necessarily because they moved away when approached, but just because they happened to be placed in other parts of the house while testing was performed. In other houses, the light was so dim that it appeared as though the birds did not notice the presence of the observer. In such cases it was impossible to discriminate between birds that were passive because they were unaware, due to the low light intensity, and those that were actually less fearful.

Not all studies indicate reduced fearfulness in hens with access to litter as adults. A study testing the effect of restricting access to the litter area for a two-week period following transfer to the

laying facility, showed that birds without access to the litter area were less fearful compared to the birds with constant access to the litter area, when tested in a tonic immobility test at 49 weeks of age (Alm et al., 2015). The authors discuss the fact that the birds with access to litter experienced a smaller drop in stocking density at the time of transfer from rearing to production, and that the effect of access to litter might have been confounded with differences in stocking density between the two groups. However, this was an experimental study with small groups of 100 birds and may not be comparable to commercial systems.

It should be mentioned that providing birds with litter, does not inevitably equate to better animal welfare. Providing birds with litter necessitates additional care as it increases the risk of diseases if not managed correctly (Sherwin et al., 2010; Lay et al., 2011). For example, the risk of acquiring infections is higher when the birds are in contact with faecal content. Vaccination regimes and ensuring that the litter is of good hygienic quality are thus of utmost importance. However, these simple husbandry procedures, if administered correctly, have the potential to improve the welfare of laying hens under commercial conditions.

5. Conclusion

In conclusion, we found that providing pullets with paper, reduced fear of a novel object as adults, but only when the birds did not have access to environmental enrichment as adults. The results of our study thus emphasise the effect of access to litter during early rearing, but also highlight the importance of provision of environmental enrichment to the adult laying hens.

Author and contributors

MB visited farms, collected data, performed data analysis, and drafted the manuscript; FMT visited farms and collected data; JN assisted with the statistical analysis; ES contributed to statistical analysis; ROM visited farms; AMJ led the project. All authors (MB, FMT, JN, ES, TBH, BR, ROM, AMJ) contributed to conception and design of the study, writing the manuscript and approved the final version.

Conflict of interest

No conflicts of interest exist in regards to this study. The funding organizations, the Foundation for Research Levy on Agricultural Products (FFL), the Agricultural Agreement Research Fund (JA) and Animalia (Norwegian Meat and Poultry Research Centre) finance applied agricultural research in collaboration with the private and public sectors. These parties' sole interest in the present study was to support publication of unbiased results in order to provide advice to poultry rearing farmers.

Ethical statement

Some Norwegian laying hen rearing farmers systematically provide day-old chicks reared in aviaries with paper on the floor of aviary rows during the first weeks of life. Others systematically avoid the use of paper as they argue that it may foul the feed belt without conferring any advantages for the chicks. After reading a detailed formal application for permission to perform a similar study (Tahamtani et al., 2016) the Animal Research Authorities ('Forsøksdyrutvalget', the Norwegian Food Authority, Norwegian Government) stated that no specific permission was needed for field studies of this type. The reason that no application is necessary for this type of study is that they simply involve systematic observation of birds that were reared and housed using common commercial procedures that are accepted by the Norwegian Food Authority. Following the study, the birds continued to be housed for egg production purposes until their depopulation at around 76 weeks of age.

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Paper IV

2   **Problem Behaviors in Adult Laying Hens – Identifying Risk Factors during**

3   **Rearing and Egg Production**

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12   Scientific section: Well-Being, and Behavior

13

14

15 **ABSTRACT**

16 Feather pecking, toe pecking, cannibalism, smothering, social clumping, hens laying eggs

17 outside the nest boxes, and reduced feather quality are examples of problem behaviors and

18 consequences reported by egg producers. The aim of this study was to identify rearing- and

19 production-related risk factors associated with producer-reported problem behaviors in

20 Norwegian layer flocks. Questionnaires were distributed to 410 egg producers nationwide, and

21 120 producers responded to the survey (response rate 29%). After exclusion of data that did

22 not comply with the instructions, the final dataset included 78 flocks (19%). The survey

23 covered questions about the farm, the flock's production results, the housing environment,

24 climate and management routines, and the behavior of the birds from 16 weeks of age until the

25 flock was euthanized at 70-80 weeks of age. The individual problem behaviors were combined

26 to generate a continuous index variable called "problem behavior", ranging from 0 (none) to 7

27 (all the listed problem behaviors) reported. Multilevel linear regression models were applied to

28 evaluate associations between the index and selected risk factors during rearing and production.

29 The primary predictor was housing system during egg production: producers with aviary flocks

30 on average (± standard deviation) reported 1.6 (± 0.60) more problem behaviors compared to

31 producers with furnished cages ($p < 0.001$). Within aviaries (n = 40), producers, on average

32 reported 1.7 (± 0.50) more problem behaviors in flocks that experienced problems with climatic

33 conditions, compared to flocks without climatic problems ($p = 0.001$). For respondents with

34 furnished cages (n = 30), on average 1.1 (± 0.50) fewer problem behaviors were reported in

35 farms with ≥ 7,500 birds compared to farms with < 7,500 birds ($p = 0.027$). In conclusion, this

36 is the first study assessing management and housing factors during the rearing and laying phase

37 associated with problem behaviors as reported by Norwegian egg producers. As this study

38 relied on producer reported observations, future studies are needed to investigate whether

39 objective measurements can verify these results.

40

41   **Key words**: problem behavior; welfare; laying hen; rearing; production

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43

INTRODUCTION

45 Laying hens (*Gallus gallus domesticus*) in commercial egg production are often housed in

46 groups much larger than the stable social groups formed in the wild (Väisänen et al., 2005),

47 and their ability to perform food search or foraging is largely dependent on the housing system

48 (Schütz and Jensen, 2001). The egg industry has tried to adapt according to increased

49 knowledge of laying hens' needs. The European ban on conventional battery cages

50 implemented from 2012 is an example (European Commission, 1999). Nevertheless, feather

51 pecking, toe pecking, cannibalism, reduced feather quality, smothering, social clumping and

52 hens laying eggs outside the nest boxes are examples of problem behaviors or consequences of

53 such behaviors which still cause concern (Brunberg et al., 2014). Problem behaviours are

54 defined as behaviours that are problematic for the person reporting the behaviour (Mills, 2003).

55 According to Mills (2003), three categories of problem behaviours have been classified: a)

56 behaviours that have adaptive value for the given species, but that are inconvenient for the

57 keeper; b) behaviours that are attempts to behave in an adaptive way in an environment that

58 does not allow for complete adaptation; and c) behaviours that express disruption of the

59 nervous system. Eggs laid outside the nest boxes increase the labour cost for the farmer and

60 are, therefore, mainly a problem for the producer. The majority of the problems reported by

61 egg producers, however, not only result in negative economic consequences but also

62 compromise laying hen welfare (Waiblinger et al., 2006). The causes of problem behaviors are

63 multifactorial. Genetic predisposition (Hughes and Duncan, 1972; Rodenburg et al., 2008),

64 early rearing conditions e.g. environmental complexity (Janczak and Riber, 2015; Brantsæter

65 et al., 2016a; Brantsæter et al., 2016b; Brantsæter et al., 2017), stockmanship and management

66 procedures e.g. access to pecking substrate (Blokhuis and Wiepkema, 1998; Tahamtani et al.,

67 2016) are among the influencing factors. Irrespective of the cause, problem behaviors may

68   indicate that the birds' needs are not fulfilled and might therefore serve as indicators of

69   suboptimal welfare.

70

71   Egg production practices differ between countries making it difficult to extrapolate findings in

72   one country or region of the world to others. There are several different hybrids and strains of

73   laying hens commercially available. In 2015, 70% of the layers in Norwegian egg production

74   were Lohmann layers (Lohmann Tierzucht, 2014), and the remaining 30% were Dekalb White

75   (Hendrix Genetics, 2014). Ninety-seven to 98% of hens used in Norwegian commercial egg

76   production are white strains (Lohmann LSL and Dekalb White) while Lohmann Brown and

77   ISA Brown constitute the remaining 2–3%. On a global scale, the health status of Norwegian

78   layer flocks is exceptionally good, and the only vaccines administered routinely are against

79   Marek's disease and coccidiosis (Griffiths, 2016). In addition to legislation controlled by the

80   European Union, Norwegian producers have to conform to specific, strict national laws and

81   regulations concerning animal welfare. As an example beak trimming has been banned in

82   Norway since 1974 (Frøslien, 1997) whereas internationally, although the EU will implement

83   similar rules shortly, most flocks are still beak trimmed. Another example is that, unless the

84   farm already had a higher number of birds when the law was founded (in 2004), the maximum

85   farm size allowed by national legislation is 7,500 birds (Landbruks- og matdepartementet,

86   2001). Norwegian layer flocks are thus small compared to other European countries such as

87   Sweden (23,000 birds per farm) (Svenska ägg, 2015) and Belgium (27,000 birds per farm)

88   (Stadig et al., 2015). In 2015, the number of registered egg production farms nationwide with

89   flocks ≥ 1,000 birds was 585 (Bagley, 2016). Furthermore, Norway is one of the few countries

90   worldwide where the majority of adult layers are kept in loose-housed systems (European

91   Commission, 2011; EC-CIRCABC, 2014; Landbrug og Fødevarer Erhvervsfjerkræsektionen,

92   2015)Landbrug og Fødevarer Erhvervsfjerkræsektionen, 2015). In Norway, the vast majority

of loose-housed birds are kept in indoor multi-tier aviary systems, while only 15 (2.5%) of the

585 egg producers have single-tier floor systems (Bagley, 2016). A single-tiered floor system

has litter areas along the outer walls and an elevated slatted area with feed, water, perches and

nest boxes along the middle. The option to move between different heights provides birds in

aviaries with more available space and increases environmental complexity compared to

housing in single-tier systems. The aviary systems consist of aviary rows and corridors. For

loose-housed systems, Norwegian legislation specifies that at least a third of the floor (equal to

250 cm^2 per bird) should contain litter (Landbruks- og matdepartementet, 2001). The aviary

corridors provide the birds with available space and litter (sawdust) where the birds can

perform highly motivated behaviors such as feed searching behavior, pecking, scratching and

dustbathing. The aviary rows usually have three tiers. Drinking nipples and food troughs run

along the bottom and middle tier of the aviary row, while the top tier contains perches. Nest

boxes are positioned inside the aviary rows, but the exact location of the nest boxes depend on

the specific aviary design. The main differences between rearing aviaries and production

aviaries are that the rearing aviaries lack nest boxes, the group size during rearing is larger, and

the stocking density is higher compared to aviaries used for egg production. Also, in production

aviaries the hens have access to litter at all times. However, during the first weeks of rearing,

the chicks are confined inside the aviary row to ensure they have easy access to the food and

water in the aviary rows. In 2016, 56% of adult layers were housed in aviary systems, 39% in

furnished cages and 5% of egg producing flocks were in organic systems (Karianne Fuglerud

Ingerød (Norwegian Poultry Association, personal communication)). However, most layer

pullets in Norway (80%) are reared in aviaries (Nils Steinsland (Steinsland AS, personal

communication)) so a minority of the aviary-reared birds are inevitably transferred to furnished

cages, rather than production aviaries at the beginning of the laying phase. Confined housing

systems are either furnished cages (maximum ten birds per cage) or colony cages (up to 100

118 birds per cage; Rodenburg et al., 2005). Of the 39% of egg production farms with confined

119 housing systems in Norway, the majority keep the birds in furnished cages, with a maximum

120 of nine hens per cage (Bagley and Rædergård, 2016). Each furnished cage contains a nest box,

121 a designated dustbathing area on top of the nest box and two perches. Water pipes with drinking

122 nipples run along the back wall of the cages. The feed trough runs along the front of the

123 furnished cages. The wire mesh floor is slightly tilted to ensure eggs roll down onto the egg

124 collection belt below the feed trough. The Norwegian legislation dictates that each hen should

125 have access to at least 850 cm² of cage area (Landbruks- og matdepartementet, 2001).

126

127 During early life leaving beaks intact, access to perches and age at transfer from the rearing

128 farm to the production farm are among the factors important for laying hen welfare (Janczak

129 and Riber, 2015; Tahamtani et al., 2016; Brantsæter et al., 2017). Development of problem

130 behaviors can be influenced by factors during hatching, rearing or at the egg production farm.

131 As an example, studies investigating risk factors for reduced plumage quality in adult layer

132 flocks, identified feather pecking during rearing as a risk factor (Zeltner et al., 2000; Bestman

133 et al., 2009; Gilani et al., 2013; de Haas et al., 2014). Other studies report that early access to

134 perches during the rearing period reduced both cloacal cannibalism and the prevalence of floor

135 eggs during the production period (Gunnarsson et al., 1999). The birds are transferred from the

136 rearing farm to the egg producer at 15-16 weeks of age. A determining factor for the ability to

137 cope with this potentially stressful event is the housing system during rearing. After transfer

138 from the rearing farm, aviary-reared birds displayed more alert behavior towards an object,

139 started laying eggs earlier (Tahamtani et al., 2014), and were less fearful 19, 21 and 23 weeks

140 of age (Brantsæter et al., 2016a; Brantsæter et al., 2016b) compared to cage-reared birds. Any

141 cause of stress during the production period can increase the risk of problem behaviors (El-

142 Lethey et al., 2000). Factors previously found to increase the risk of problem behaviors are

143   fluctuating indoor climate (i.e. uneven temperature or draft (Channing et al., 2001)), lack of

144   environmental enrichment (Zeltner et al., 2000; Tahamtani et al., 2016; Brantsæter et al., 2017),

145   incorrect feed texture (Van Krimpen et al., 2005) and suboptimal quantity and quality of human

146   contact (Coleman and Hemsworth, 2014). Any supplement in addition to feed and water, which

147   encourages active, explorative or foraging behavior, is considered 'environmental enrichment'.

148   Examples of environmental enrichment supplied by egg producers are empty plastic boxes,

149   box lids, toy balls, old CDs, pecking stones (aerated concrete and calcium silicate hydrate

150   blocks), sawdust, oyster shells, and cut up pieces of manure belts or egg belts (Brantsæter et

151   al. 2017).

152

153   As most problem behaviors (i.e. feather pecking, cannibalism and floor eggs) are discovered

154   after the birds reach a certain age, these problems are primarily of concern to egg producers.

155   The main aim of this study was to identify rearing- and production-related risk factors

156   associated with producer-reported problem behaviors in Norwegian layer flocks.

157

158   **MATERIALS AND METHODS**

159   *Study Design and Data Collection*

160   The questionnaire was designed, constructed and distributed using the online software provided

161   by Questback[TM] (www.questback.com). The questionnaire was divided into four different

162   parts: 1) General questions about the farm; 2) Questions about the flock's production results;

163   3) Questions about the environment, climate and management routines; and 4) Questions

164   regarding the observed behavior of the birds from arrival at the egg production farm until the

165   flock was euthanized at 70-80 weeks of age.

166 Questions were a combination of multiple-choice (some of which the respondent had to choose

167 one option and others where it was possible to tick several options) and open-answer questions.

168 The respondents were routed to follow-up questions relevant to their respective production

169 system and the behaviors they reported to have observed. Respondents were instructed to reply

170 for their current flock if the animals were over 60 weeks of age or for their previous flock if

171 their present flock was younger than 60 weeks of age. If the respondent had several flocks

172 simultaneously, he/she was asked to respond for one flock only.

173 E-mail addresses were acquired from egg-packing centers throughout Norway. During the

174 design of the questionnaire, it was sent to industry advisors and a selection of egg producers

175 for testing to ensure the quality and relevance of the questions. The survey was distributed to

176 all the egg producers whose e-mail addresses we acquired during the data collection period

177 (August – November 2015). Throughout the data collection period the egg producers who had

178 not yet responded to the questionnaire were sent biweekly e-mail reminders until the

179 questionnaire was registered as "completed" by the Questback system or the period of data

180 collection was stopped. The questionnaire, in Norwegian, is available from the corresponding

181 author on request.

182

183 *Categorization of Explanatory and Outcome Variables*

184 After completion of the data collection, the data were quality controlled manually in Microsoft

185 Excel (2013) to make sure that the respondent had replied according to the instructions.

186 Inclusion criteria were that the respondents had answered for only one flock and that the age

187 of the given flock was minimum 60 weeks of age. Open answer data had to be labeled and

188 coded appropriately for statistical analysis. Categorization of continuous variables or merging

189 of categories was necessary for further analysis was done with utmost care to ensure that the

190 categories were biologically relevant. For example, regarding housing system at the egg

191 production farm, the category named "other system" included aviaries with outdoor access,

192 organic systems, and floor systems. Because these systems differ substantially from a

193 conventional aviary regarding the level of environmental stimuli, it was considered better to

194 keep them separate from the "aviary" group.

195 The outcomes included in the questionnaire were feather pecking (gentle and severe), toe

196 pecking, cannibalism, social clumping, hysteria/panic, floor eggs and feather quality. The

197 definitions given to the producers in the questionnaire are listed in Table 1. Before data

198 management, the outcomes were coded as binomial variables (0 = behavior not reported; vs. 1

199 = behavior reported by respondent). Feather quality was also categorized as a binomial

200 variable, where 0 = good plumage quality at ≥ 60 weeks of age; vs. 1 = reduced plumage quality

201 at ≥ 60 weeks of age reported by respondent. A continuous outcome index variable was

202 generated to avoid multiple testing of seven different outcome variables against a large number

203 of explanatory variables. The first step in creating the index was to tabulate the outcome

204 variables by each explanatory variable. The tabulation was conducted to evaluate whether any

205 outcome variables were not reported by the respondents and should therefore not be included

206 in the index.

207

208 ***Statistical Analysis***

209 All statistical analyses were conducted using Stata SE 14 (StataCorp LP). P-values ≤ 0.05 were

210 considered statistically significant. In the first step of model building, all explanatory variables

211 were screened individually to assess their association with the index variable. The screening

212 was conducted by multilevel mixed-effects linear regression, with rearing farmer as a random

213 effect. Only explanatory factors with $p < 0.2$ were kept for step two which was backward

214   stepwise selection. Log likelihood tests were used to assess the overall significance of

215   categorical variables with more than two levels.

216   The final model, also with rearing farmer as a random effect, was selected based on backward

217   stepwise selection. Only factors with $p < 0.1$ were retained in the final model. The final model

218   was tested to ensure it conformed to the assumptions of linear regression (normality of residuals

219   and homogeneity of variance) by inspection of the Q-Q plot and by the Shapiro-Wilk test and

220   by plotting standardized residuals versus fitted values. Results from the models are presented

221   as β coefficient ± standard error.

222   Based on the results from the whole dataset, a decision was made to analyze subsets for each

223   housing category, i.e. aviary systems and furnished cages. The models for these subsets were

224   constructed according to the same procedure as described for the dataset as a whole.

225

226   **RESULTS**

227   *Study Population*

228   The response rate achieved in our study was 29% (n = 120/410). However, after exclusion of

229   replies that did not conform to the inclusion criteria, the final dataset included 78/410

230   respondents (19%). The 78 flocks were located in 17 out of 19 Norwegian counties, ranging

231   from one to 19 respondents per county. Fourteen (18%) of the respondents replied for flocks

232   with less than 7,500 birds, whereas 64 (82%) flocks contained at least 7,500 birds. Both of the

233   major egg-laying hybrids in Norway were represented: 55 respondents (71%) had Lohmann

234   layers, and 23 egg producers (29%) had Dekalb White. No producers had ISA Brown layers

235   and only four of 78 producers reported that they kept Lohmann Brown layers. However, these

236   producers had mixed flocks of Lohmann Brown and Lohmann LSL and the proportions of

237   white versus brown birds in these flocks was not recorded. The final dataset included flocks

238   delivered by 13 of 16 possible rearing farmers nationwide, whereby each rearing farmer

239   contributed with one to 14 flocks. Fifty (64%) of the 78 flocks were reared in aviaries, 15 (19%)

240   of the flocks were reared in cages, and for the remaining 13 (17%) of the flocks, the rearing

241   conditions were unknown.

242

243   The distribution of the problem behavior index per housing system is presented in Figure 1.

244   Due to the low number of observations (n = 8), and the heterogeneity of the flocks grouped as

245   "other system", this group was not analyzed further, but separate models were built for the

246   aviary producers (n = 40) and furnished cage producers (n = 30).

247   The 40 respondents with aviary housing systems were located in 16 of the 19 Norwegian

248   counties. Eleven different rearing farmers were represented, each contributing with one to eight

249   flocks. The 30 respondents with furnished cage systems represented egg producers from 11 out

250   of the 19 Norwegian counties. Nine different rearing farmers were represented, each

251   contributing with one to seven flocks. The number and percentage of aviary and furnished cage

252   respondents within each of the explanatory variable levels used to investigate the association

253   with reported problem behaviors are shown in Tables 2A-D.

254

255   ***Data Management and Multilevel Linear Regression Models***

256   In the final dataset (n = 78) all the behavioral outcomes were reported by at least 14 respondents

257   (Table 1). The generated behavior index thus included all seven behaviors and ranged from 0

258   (none of the behaviors) to 7 (all the problem behaviors) reported by each respondent. The

259   frequency of each outcome variable, grouped by each predictor is presented in the

260   supplementary material (Tables S1-8). The explanatory variables associated with the index

261   variable (p < 0.2; highlighted as bold in the Table 3A-D) were included when starting the

262   backward stepwise reduction of the multilevel linear regression model for the final dataset.

263   Two factors were related to the rearing period and nine factors related to the egg production

264   farm (Table 3A-D).

265

266   ***Full Model.*** After backward stepwise reduction where only explanatory variables with $p < 0.1$

267   were retained, the final model for the behavior index contained housing at production farm

268   (furnished cages compared to aviaries), challenges with climatic conditions at the production

269   farm (yes/no) and hybrid (Lohmann LSL versus Dekalb White). The frequency of producer-

270   reported problem behaviors was higher in aviaries compared to furnished cages (β coefficient

271   ± standard error; 1.61 ± 0.36; $p \leq 0.001$) and was greater if the birds had been exposed to

272   problems with climatic conditions during lay (1.24 ± 0.38; $p = 0.001$). Also, there was a

273   tendency for fewer reported problem behaviors among the respondents with Dekalb layers

274   compared to Lohmann layers (-0.65 ± 0.37; $p = 0.083$). No variation was explained by the

275   random effect rearing farmer ($p = 1.00$). The assumptions of linear regression were fulfilled.

276

277   ***Aviary Model***. After backward stepwise removal of explanatory factors with $p > 0.1$ (Table 4),

278   the best model for the subset of aviary flocks (n = 40) included only the variable "challenges

279   with climatic conditions" (yes/no). The direction of effect was the same as for the dataset as a

280   whole; flocks with reported problems with climatic conditions during lay scored higher on the

281   index variable, suggesting more problem behavior, compared to flocks that did not experience

282   problems related to climatic conditions (1.70 ± 0.50; p = 0.001). No variation was explained

283   by the random effect rearing farmer (p = 0.21). The assumptions of linear regression were

284   fulfilled.

285

13

286 ***Furnished Cage Model.*** After backward stepwise removal of explanatory factors with $p > 0.1$

287 (Table 5) the best model for the furnished cage subset (n = 30) included number of animals at

288 the farm (< 7,500 versus ≥ 7,500 birds) and use of a LUX-meter to control the light intensity

289 in the hen house (yes/no). The results indicate that more problem behavior was reported in

290 farms with less than 7,500 birds compared to farms with at least 7,500 birds (-1.42 ± 0.51; $p <$

291 0.01). Also, there was a tendency for lower risk of observing problem behaviors among the

292 respondents who used a LUX-meter to adjust the light intensity (-0.82 ± 0.45; $p = 0.07$). No

293 variation was explained by the random effect rearing farmer ($p = 0.16$). The assumptions of

294 linear regression were fulfilled.

295

296   **DISCUSSION**

297 ***Summary of Main Findings***

298 The aim of this study was to identify rearing- and production-related risk factors associated

299 with producer-reported problem behaviors in Norwegian layer flocks. As all the seven

300 outcomes included in the survey were reported, a continuous index variable was created.

301 Overall, egg producers with aviary systems reported more problem behaviors compared with

302 furnished cage producers. Additionally, issues with climatic control during lay were associated

303 with increased observation of problem behaviors. There was a tendency for the producers with

304 Dekalb layers to report fewer problem behaviors compared to the producers with Lohmann

305 layers. The main risk factor associated with observing more problem behaviors in aviary

306 systems was issues with climatic control during lay. For furnished cages, producers with

307 smaller farms reported more problem behaviors compared to larger farms, while those who

308 used a LUX-meter to adjust the light intensity in the hen house tended to observe fewer problem

309 behaviors.

310

Risk factors Related to the Production Phase

Housing System. A key finding in our study was that aviary producers reported more problem behaviors than the furnished cage producers did. Loose-house systems enable birds to express more behaviors compared to birds in confined housing (reviewed by (Lay et al., 2011)). Most of the behavioral problems reported in this study, except eggs laid outside the nest boxes, are behaviors that are also observed in wild jungle fowl. However, the consequences of these behavioral responses might differ for birds kept in commercial housing conditions compared to animals living in the wild. As an example, fear-related responses (e.g. the birds' responses when faced with predators) can increase the chance of survival in the wild. On the contrary, commercial housing conditions may not allow the birds to avoid the fear-inducing stimuli, and fear-responses might even result in injuries or suffocation if the birds fly/run into the metal constructions or pile on top of each other (Jones, 1996). This example illustrates how behavioral responses with adaptive value in the wild can compromise animal welfare and the farmer's economy in commercial egg production (Mills, 2003). Birds in aviaries have the possibility to express more behaviors than birds in furnished cages. Hence, it will also be easier for aviary birds to perform some of the problem behaviors (e.g. laying eggs in the litter rather than in the designated nest boxes) than for cage-housed birds. Our results indicate that this aspect of Norwegian egg production is comparable to results from studies conducted in other countries, where aviaries are also associated with higher proportions of problem behaviors compared to furnished cages (Rodenburg et al., 2005; Tauson et al., 2006; Sherwin et al., 2010; Shimmura et al., 2010; Lay et al., 2011; Stadig et al., 2016). Animal welfare, conceptualized as a continuous scale from poor to good, is secured when the animal is healthy, experiences positive rather than negative affective states and is able to express innate behaviors (Fraser et al., 1997). Thus, without additional measures of welfare, caution should be exercised when

335 using the current study to argue that one housing system might be better for animal welfare

336 than another.

337

338 Our finding of more problem behaviors reported in the aviaries compared to the furnished cages

339 might reflect the actual situation. However, the results could be explained by the limitations of

340 the data collection method (questionnaire) (see paragraph under methodological

341 considerations). Furthermore, egg producers with furnished cage systems might be different

342 from aviary egg producers regarding their motivation to focus on these problems, their level of

343 awareness or possibility to detect these problem behaviors. For instance, if eggs are laid outside

344 a nest box in a furnished cage, the tilted floor will allow the egg to end up in the egg belt

345 without increased labor of the farmer. Regarding assessment of toe pecking, feather pecking or

346 plumage quality, the location of the furnished cage (top, bottom or low tier) might influence

347 the farmers' ability to physically assess the feathers while walking through the house (Tablante

348 et al., 2000; Brantsæter et al., 2016a). Similarly, assessment of the number of birds perching

349 on the top tier of the aviary might be a challenge for aviary producers.

350

351 ***Issues with Climatic Conditions.*** Our results of the association between climatic conditions

352 and more problem behaviors reported by the aviary respondents thus add support to existing

353 knowledge. Among the aviary producers who described problems controlling the climatic

354 conditions in the henhouse, issues with maintaining stable and optimal temperatures, uneven

355 temperature in different parts of the house and draft were most commonly reported. A possible

356 explanation for the association between climatic conditions and increased risk of problem

357 behaviors can be the altered stocking density when uneven temperatures cause birds to cluster

358 in some parts of the house and avoid other areas. The effect of distorted stocking density in

loose-housed systems is not entirely understood as some, but not all studies report increased risk of aggression and reduced feather quality with increased stocking density (Gunnarsson et al., 1999; Gunnarsson et al., 2000; Channing et al., 2001; Nicol et al., 2006; Collins et al., 2011; Widowski et al., 2016). Clustering should be taken seriously, as it affects the birds' thermoregulatory abilities (Green and Xin, 2009), and heat stress has been demonstrated to cause immunosuppression in adult layers (Mashaly et al., 2004).

Besides, in areas with clustering, or reduced ventilation, there can be "blind spots" with build-up of gasses such as ammonia and carbon dioxide. As exposure to these gasses is uncomfortable, they possibly reduce the time the stockperson spends in the hen house. Ammonia exposure can cause health problems not only among the animals but also be negative for the stockpeople (Kirkhorn and Schenker, 2002; Kirychuk et al., 2003; Xin et al., 2011; David et al., 2015a; David et al., 2015b). In laying hen houses with confined cage systems, researchers concluded that areas with low ventilation were more common in corners and along the back of the house, posing a potentially bigger problem for the stockpeople than the animals (Prodanov et al., 2016). In comparison, in loose-housed systems, where the birds have access to the areas with potentially increased concentration of harmful gasses, this is a greater concern regarding animal welfare. On the contrary, as opposed to loose-housed hens, birds kept in cages are not be able to avoid areas of suboptimal climatic conditions. In other words, issues with climatic conditions might affect loose-housed and birds housed in confined cages differently.

Management and Stockmanship Differences between Aviary and Furnished Cage Producers. The association between issues with climatic control and increased occurrence of problem behaviors as perceived by the egg producers might be real. However, from our study, we cannot exclude the possibility that climatic control is a reflection of the general

17

383 management. The result that use of a LUX-meter tended to be associated with a decrease of

384 reported problem behavior among furnished cage producers could be another indication of

385 management differences between the aviary and furnished cage producers. Loose-housing

386 systems are considered more challenging to manage, and demand a better stockmanship

387 compared to confined housing systems (see review (Appleby and Hughes, 1991; Häne et al.,

388 2000)). The effect of the stockpeople can therefore be of greater importance for loose-housed

389 birds compared to cage-housed birds. The majority of the respondents never exceeded one hour

390 of daily inspections, regardless of whether the birds had recently arrived at the farm or were

391 around the age of the onset of lay (Table 3C). Towards the end of the production phase, most

392 of the respondents spent less than 30 minutes inside the house. The aviary producers inspected

393 the birds more often, compared to the furnished cage producers during the onset of lay as well

394 as later in the production cycle (Table 3C). However, from our data is it not possible to

395 distinguish what came first, the problem behaviors and therefore a need for more frequent

396 inspections, or whether the more frequent inspections allowed the aviary respondents to detect

397 the problems better than the furnished cage producers. Furthermore, lack of positive interaction

398 with a human can be a cause of fearfulness and stress in laying hens (Edwards et al., 2013). In

399 our study, no direct measures of the quality of farmer-animal interactions or fearfulness were

400 obtained, so the bird's association of human presence as a positive or negative event is

401 unknown. Fearfulness is associated with several of the outcomes covered by the questionnaire,

402 specifically feather pecking (Uitdehaag et al., 2009; de Haas et al., 2010; de Haas et al., 2013;

403 Kops et al., 2013; Rodenburg et al., 2013), cannibalism (Newberry, 2004) and smothering

404 (Bright and Johnson, 2011; Barrett et al., 2014). If the animals in our study associated the

405 farmer with a negative event (i.e. danger), the increased number of inspections might have been

406 among the causative factors for the increased occurrence of problem behaviors perceived by

407 the aviary respondents. In our study, furnished cage producers reported less problem behavior

18

408   in farms with ≥ 7,500 birds compared to farms with < 7,500 birds (Table 5). As the time the

409   farmer spent inspecting the birds was a maximum of 30 - 60 minutes irrespective of the number

410   of birds per cage, we cannot rule out the possibility that the egg producer to a larger degree

411   overlooked problem behaviors or consequences of such behaviors in farms with more birds.

412   Another possible explanation for this association is that a bigger farm could reflect a more

413   dedicated and professional producer compared to a small farm. Fifty of the 78 (64%) producers

414   had at least seven years of experience with egg production. As the maximum number of birds

415   per farm has been 7,500 since 2004, it is unlikely that lack of experience is confounded with

416   the effect of flock size. Future studies are required to assess the potential confounding effect

417   of management associated with the identified risk factors.

418

419   ***Genetics as a Risk Factor***

420   Hybrid was included as an explanatory variable in the linear regression model for the whole

421   dataset (Table 3A). There was a tendency (p = 0.083) for fewer observed problem behaviors

422   among Dekalb producers compared to Lohmann producers. To a certain extent, this supports

423   anecdotal evidence from egg producers who have the impression that Lohmann layers more

424   often struggle with feather pecking and reduced feather quality compared to Dekalb layers

425   (Brunberg et al., 2014). On the other hand, Dekalb producers more often consider floor eggs to

426   be a problem. Although White and Brown layer strains have been found to differ concerning

427   fearfulness (Uitdehaag et al., 2011), propensity to feather peck and develop cannibalistic

428   behavior (Kjaer and Sorensen, 2002), the authors are not aware of studies focusing on

429   differences in problem behaviors between Lohmann and Dekalb layers. Future studies are

430   needed to test whether the tendency detected in our study is replicable in an observational

431   study, as the finding based on our questionnaire also could be caused by subjective bias (see

432   paragraph under methodological considerations).

433

434   ***Rearing-related Risk Factors***

435   Thirteen of the 78 egg producers (17%) did not know if their flock was reared in confined or

436   loose-housed systems (Table 3A). The majority of the producers that did not know the rearing

437   conditions of their birds had furnished cage systems (Table 2A). Possible explanations are that

438   furnished cage producers are not as familiar with the different housing systems rearing farmers

439   can utilize during rearing, there may be lack of knowledge of the effect of rearing under

440   different conditions, or they may trust the rearing farmer with the decision. From this, one

441   could question whether producers with furnished cages do not have the same interest, or

442   perceived need to be informed, about rearing effects, as producers with aviary systems. Rearing

443   farmer was included in the multilevel linear regression models as a random effect to deal with

444   the fact that flocks from the same rearing farmer may be more similar than flocks from different

445   rearing farmers. However, rearing farmer was not identified as a source of variation in the full

446   model, the aviary subset or the furnished cage subset.

447

448   ***Methodological considerations***

449   The primary methodological consideration of this study is the possible systematic error

450   introduced because the data relies on producer-perceived information. The producer is an

451   unmeasurable confounder (Dohoo, 2014) as he or she both affects the exposure (i.e.

452   management of the flock) and the outcome (i.e. is the one who reported the behaviors).

453   Furthermore, the questionnaire is sensitive to information bias (e.g. recall bias,

454   misinterpretation of the questions or different opinions of the provided definitions of the

20

455 behaviors) (Dohoo, 2014). This study was retrospective relying on respondents' memory or

456 written records. Depending on the age of their current flock, some respondents may have had

457 to recall from up to a year back. The data collected does not provide information about whether

458 replies were based on objective measures or subjective impressions (e.g. of levels of ammonia).

459 Finally, we cannot rule out the possibility of selection bias due to non-response bias (Dohoo,

460 2014), as we did not follow up the non-responders to see whether their replies would have

461 differed from responders. Non-response bias can contribute to higher reports of problem

462 behaviors, as producers without experience with these issues might have lacked the motivation

463 to complete the questionnaire. Non-response bias could also result in under-reporting of these

464 problems if producers experiencing these problems did not reply to the questionnaire.

465

466 *Validity*

467 The aim of the current study was to assess risk factors, rather than detect the prevalence of

468 problem behaviors. Estimating prevalence would have required a different study design based

469 on random selection of egg producers. The following paragraph therefore focuses on

470 limitations in the assessment of the risk factors identified in the current study. Generalization

471 of the findings is discussed regarding internal validity (i.e. if the results are representative of

472 the source population) and external validity (i.e. if the results are valid for the target

473 population).

474

475 *Internal validity.* Selection bias arises when the study group (sample) is not representative of

476 the source population (Dohoo, 2014). The behavioral problems covered by the survey usually

477 are not present in young birds. The exclusion of flocks aged < 60 weeks was necessary to

478 ensure that the flocks were comparable. Whether this exclusion of 42 flocks introduced

479   selection bias is unknown. It cannot be ruled out that farmers replying for flocks < 60 weeks,

480   did so because they did not understand the instructions or were different from farmers who did

481   understand the instructions of the questionnaire and replied for flocks of minimum 60 weeks

482   of age.

483

484   ***External validity.*** The total number of e-mail addresses we obtained (source population) was

485   410 (out of 585 registered egg producers (target population)). The reduction of e-mail addresses

486   was due to egg-packing centers (rather than producers) not willing to cooperate, so the source

487   population is thus unlikely to differ from the target population (all Norwegian egg producers).

488   The response rate of 29% is acceptable for a rather lengthy questionnaire relying on response

489   through an electronic system (reviewed by (Sheehan, 2001)). The final dataset represents

490   nearly all the Norwegian counties, the majority of the rearing farmers and the two commercial

491   hybrids. Additionally, the proportion of respondents with aviaries and furnished cage systems

492   were close to the numbers registered by the industry (Karianne Fuglerud Ingerød (Norwegian

493   Poultry Association, personal communication).

494

495   As mentioned in the introduction, some aspects of the Norwegian egg industry are markedly

496   different from other countries (i.e. smaller farm size, small cage units with maximum nine birds

497   per furnished cage, no beak trimming and the majority of birds are loose-housed). As the risk

498   factors identified in the current study were essentially management related, their effect under

499   different farm sizes is difficult to predict. Furthermore, management regimes at larger farms

500   may be qualitatively different from management at smaller farms. The results of the current

501   study should therefore be interpreted with caution when extrapolating to other countries.

502   However, as the genetic material is the same for all who import Lohmann or Dekalb/ISA layers,

503 our findings may be of relevance to others using these breeds for egg production. Particularly

504 with the impending ban on beak trimming, the results of this study could be valuable to egg

505 producers in Europe to make them aware of potential risk factors.

506

507 *Conclusion*

508 To our knowledge, this is the first time the producer perceived occurrence of problem behaviors

509 is investigated in Norwegian egg production. All the seven outcomes covered by the

510 questionnaire (gentle feather pecking, severe feather pecking, toe pecking, cannibalism, social

511 clumping, mislaid eggs and reduced plumage quality) were reported by 18 – 60% of egg

512 producers. The main factors associated with increased risk of observation of problem behaviors

513 in our survey were problems with climatic conditions and the housing system during lay. For

514 respondents with furnished cages, the main predictor was the size of the farm: more problem

515 behaviors were reported in smaller farms compared to bigger farms. Future studies

516 investigating the causal relationships between rearing and production related risk factors and

517 problem behaviors are warranted and should include objective measures of climatic conditions

518 as well as behavioral observations by trained observers.

519

520 **ACKNOWLEDGEMENTS**

525

Ethical Statement

There was no need for approval from an ethical committee for conducting this survey as it was purely observational and did not involve any biological manipulation of the birds.

Author contributions

MB participated in the design of the questionnaire, contacted producers, collected the data, manually controlled the raw data, performed data analysis and drafted the manuscript. JN assisted in the statistical analysis and drafted the manuscript. TBH participated in the design of the questionnaire, contacted the egg-packing centers, contacted producers, assisted in the manual control of the raw data and drafted the manuscript. KM participated in the design of the questionnaire, assisted with data analysis and drafted the manuscript. AN assisted in data analysis and drafted the manuscript. ROM participated in the conceptualization and design of the study and drafted the manuscript. AMJ led the project, participated in the conceptualization and design of the study, participated in the design of the questionnaire, and drafted the manuscript. All authors accepted the final version of the manuscript.

Conflict of Interest Statement

The authors of this manuscript have declared that no competing interests exist. Animalia (Norwegian meat and Poultry Research Center) contributed with funding for the project and the author TBH is employed by Animalia. She contributed as described under author contributions. Animalia finances and performs applied agricultural research in collaboration with the private and public sectors. Animalia's sole interest in the present study was to support publication of the unbiased results in order to provide advice to poultry rearing farmers and

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750 **Table 1.** An overview of outcomes included in the questionnaire and the information the producers

751 were provided to answer the questions. The raw data is presented as the number (%) of respondents

752 (N = 78) reporting the given behavior in their most recent flock ≥ 60 weeks of age.

Behaviors included in the questionnaire	Description available to the producers when responding to the questionnaire	Number (%) of respondents who reported the given behavior
Gentle feather pecking	Gentle pecking at, or removal of feathers from own plumage or from a conspecific	41 (53%)
Severe feather pecking	More intense pecking at conspecific which causes skin lesions	40 (51%)
Toe pecking	Pecking at their own toes with resulting skin lesions	14 (18%)
Cannibalism	Pecking at conspecific resulting in big lesions in the skin, profuse bleeding, dysfunctional / removed body parts or death	21 (27%)
Social clumping	Some, or all, the hens pile up for no apparent reason. The birds do not seem disturbed or frightened.	27 (35%)
Hysteria / panic	Some, or all, of the hens abruptly, and for no obvious reason run or fly to one end of the room. The animals appear frightened.	19 (24%)
Floor eggs	Eggs laid outside the designated nest boxes	47 (60%)
Feather quality around the time of euthanasia	1) The hens were fully covered with good quality feathers	18 (23%)
	2) The hens had small patches without feathers	36 (46%)
	3) The hens were more or less naked	24 (31%)

753

754 **Table 2A-D.** The number and percentage of aviary respondents (n = 40) and furnished cage

755 respondents (n = 30) for the different explanatory variable levels.

756

757 **Table 2A)** Number of aviary (AV) and furnished cage (FC) respondents grouped by rearing related

758 explanatory variables

Rearing related variables	AV respondents		FC respondents	
	Number	Percentage	Number	Percentage
All data	40	100%	30	100%
Rearing housing				
Rearing cages	0	0%	15	50%
Aviary rearing	39	98%	5	17%
Don't know/other system	1	2%	10	33%
Hybrid				
Lohmann White or Brown	31	78%	18	60%
Dekalb White	9	22%	12	40%
Age of transfer to producer				
< 16 weeks of age	10	25%	7	24%
16 weeks of age	24	60%	19	66%
> 16 weeks of age	6	15%	3	10%

759 AV = aviary; FC = furnished cages

760   **Table 2B)** Number of aviary (AV) and furnished cage (FC) respondents grouped by explanatory

761   variables related to the egg production farm.

Production farm related variables	AV respondents		FC respondents	
	Number	Percentage	Number	Percentage
All data	40	100%	30	100%
Experience with egg production				
< 7 years	19	48%	7	23%
≥ 7 years	21	52%	23	77%
Size of the flock				
< 7500 birds	5	12%	6	20%
≥ 7500 birds	35	88%	24	80%
Challenges with climatic conditions during production				
No	28	70%	24	80%
Yes	12	30%	6	20%
Challenges related to content or distribution of feed				
No	27	68%	22	73%
Yes	13	32%	8	27%
Light intensity adjusted using a LUX-meter				
Yes	25	62%	21	70%
No	15	38%	9	30%
Number of birds per furnished cage				
≤ 10 birds	-	-	24	80%
> 10 birds	-	-	6	20%

762   AV = aviary; FC = furnished cages

763 **Table 2C)** Number of aviary (AV) and furnished cage (FC) respondents grouped by explanatory

764 variables related to the inspection routines at the egg production farm

Production farm inspection related variables	AV respondents		FC respondents	
	Number	Percentage	Number	Percentage
All data	40	100%	30	100%
Number of stockpeople				
1-2 people	17	42%	12	40%
≥ 3 people	23	58%	18	60%
Inspections soon after delivery to producer				
1-2 times per day	11	28%	12	40%
3 times per day	13	32%	10	33%
≥ 4 times per day	16	40%	8	27%
Inspections around onset of lay				
1-2 times per day	18	45%	22	73%
≥ 3 times per day	22	55%	8	27%
Inspections after peak of lay				
1 time per day	7	18%	6	20%
2 times per day	25	62%	22	73%
≥ 3 times per day	8	20%	2	7%
Inspection time soon after delivery to producer				
< 1 hour per day	19	48%	20	67%
1-2 hours per day	15	38%	7	23%
> 2 hours per day	6	14%	3	10%
Inspection time around onset of lay				
< 1 hour per day	25	62%	22	73%
> 1 hour per day	15	38%	8	27%
Inspection time per day later in production				
< 30 minutes per day	11	28%	8	27%
30 - 60 minutes per day	22	55%	16	53%
> 60 minutes per day	7	17%	6	20%

765 AV = aviary; FC = furnished cages

766   **Table 2D)** Number of aviary (AV) and furnished cage (FC) respondents grouped by explanatory

767   variables related to use of environmental enrichment at the egg production farm.

Production farm related variables	AV respondents		FC respondents	
	Number	**Percentage**	**Number**	**Percentage**
All data	40	100%	30	100%
Access to environmental enrichment (edible and non-edible)				
Yes	38	95%	29	97%
No	2	5%	1	3%
Use of enrichment excluding shell sand and pebbles for the gizzard				
Yes	32	80%	25	83%
No	8	20%	5	17%
Types of edible enrichment the birds are given access to				
None	4	10%	4	13%
One type	12	30%	16	53%
Two types	12	30%	10	33%
> 2 types	12	30%	0	0%
Types of non-edible environmental enrichment the birds are provided				
None	9	23%	28	93%
One type	12	30%	2	7%
≥ 2 types	19	47%	0	0%
Substrate used as dustbathing material				
Saw dust	-	-	14	47%
Other	-	-	16	53%
Access to the dust bathing area before onset of lay				
Yes	-	-	18	60%
No	-	-	12	40%
Provision of dust bathing material occurs				
≥ 1 per week	-	-	11	37%
Less regularly than weekly	-	-	19	63%

768   AV = aviary; FC = furnished cages

Tables 3A-D. The number of observations, mean index score (standard deviation) and p-value for

between-level comparisons for each level of each factor included in the questionnaire (N = 78).

Values for the index variable range from 0 to 7. The p-values are the result of multilevel linear

regression models with rearing farmer as random effect with individual screening of the

explanatory variables. Variables with p-values < 0.2 (highlighted in bold) were included in

backward stepwise reduction. The levels for each explanatory variable used as reference category

are marked as "ref".

Table 3A) Number of observations, mean score (SD) and p-value for between-level comparisons

for problem behavior for each level of all factors related to the rearing phase.

Rearing related variables	Number of respondents	Problem behavior index (values 0 - 7)	
		Mean (SD)	*P*-value
Rearing housing	78		
Rearing cages	15	2.13 (0.99)	ref.
Aviary rearing	50	3.88 (1.80)	**≤ 0.001**
Other rearing system/don't know	13	3 (1.83)	**0.164**
Hybrid	78		
Lohmann White or Brown	55	3.62 (1.74)	ref.
Dekalb White or	23	2.87 (1.87)	**0.085**
Age of transfer to producer	77		
< 16 weeks of age	18	3.17 (1.82)	ref.
16 weeks of age	48	3.54 (1.83)	0.448
> 16 weeks of age	11	3.18 (1.78)	0.982

778

779 **Table 3B)** Number of observations, mean score (SD) and p-value for between-level comparisons

780 for problem behavior for each level of all factors related to the production phase.

Egg production farm variables	Number of respondents	Problem behavior index (values 0 - 7)	
		Mean (SD)	*P*-value
Experience with egg production	78		
< 7 years	28	3.57 (1.87)	ref
≥ 7 years	50	3.30 (1.76)	0.518
Production housing system	78		
Furnished cages	30	2.33 (1.35)	ref.
Aviary	40	4.18 (1.68)	**< 0.001**
Other housing system	8	3.50 (2.00)	**0.061**
Size of the flock	78		
< 7,500 birds	14	3.5 (1.51)	ref.
≥ 7,500 birds	64	3.38 (1.86)	0.812
Challenges with climatic conditions during production	78		
No	56	3.04 (1.67)	ref.
Yes	22	4.32 (1.81)	**0.003**
Challenges related to feed or feed distribution during production	78		
No	56	3.36 (1.69)	ref.
Yes	22	3.5 (2.09)	0.75
Is light intensity in the production house set using a LUX-meter	78		
Yes	51	3.18 (1.79)	**0.127**
No	27	3.81 (1.78)	ref.

781

782 **Table 3C)** Number of observations, mean score (SD) and p-value for between-level comparisons

783 for problem behavior for each level of all factors related to inspection at the egg production farm.

Inspection variables at egg production farm	Number of observations (n)	Problem behavior index mean (SD)	*P*-value
Number of stockpeople	78		
1-2 people	33	3.45 (1.58)	ref.
≥ 3 people	45	3.33 (1.95)	0.711
Inspections soon after delivery to producer	78		
1-2 times per day	24	2.92 (1.80)	ref.
3 times per day	24	3.63 (1.79)	**0.162**
≥ 4 times per day	30	3.60 (1.80)	**0.155**
Inspections around onset of lay	78		
1-2 times per day	42	3.00 (1.55)	ref.
≥ 3 times per day	36	3.90 (1.97)	**0.029**
Inspections after peak of lay	78		
1 time per day	13	3.23 (1.64)	ref.
2 times per day	54	3.22 (1.76)	0.987
≥ 3 times per day	11	4.45 (1.97)	**0.085**
Inspection time soon after delivery to producer	78		
< 1 hour per day	41	3.10 (1.81)	ref.
1-2 hours per day	23	3.78 (1.57)	**0.134**
> 2 hours per day	14	3.64 (2.06)	0.316
Inspection time around onset of lay	78		
< 1 hour per day	49	3.24 (1.61)	ref.
≥ 1 hour per day	29	3.66 (2.07)	0.324
Inspection time per day later in production	78		
< 30 minutes per day	21	3.24 (1.64)	ref.
30 - 60 minutes per day	41	3.44 (1.87)	0.674
> 60 minutes per day	16	3.5 (1.90)	0.658

784

785 **Table 3D)** Number of observations, mean score (SD) and p-value for between-level comparisons

786 for problem behavior for each level of all factors related to administration of environmental

787 enrichment at the egg production farm.

Environmental enrichment at egg production farm	Number of observations (n)	Problem behavior index mean (SD)	*P*-value
Access to environmental enrichment (edible and non-edible)	78		
Yes	75	3.45 (1.78)	**0.162**
No	3	2.00 (2.00)	ref.
Use of enrichment excluding shell sand and pebbles for the gizzard	78		
Yes	63	3.43 (1.81)	0.752
No	15	3.23 (1.80)	ref.
Types of edible enrichment the birds are given access to	78		
None	8	2.88 (1.73)	**0.133**
One type	30	3.13 (1.87)	**0.102**
Two types	23	3.48 (1.56)	0.35
> 2 types	17	4 (1.97)	ref.
Types of non-edible environmental enrichment the birds are provided	78		
None	42	2.79 (1.57)	**≤ 0.001**
One type	14	3.57 (1.83)	**0.114**
≥ 2 types	22	4.45 (1.74)	ref.

788

789 **Table 4**. Overview of the five explanatory variables with p < 0.2 that were relevant to include in

790 analysis for the aviary (AV) flocks (n = 40). Mean and standard deviation (SD) are described for

791 each level.

Explanatory variables (AV)	Number of observations	Problem behavior index mean (SD)	*P*-value
Challenges with climatic conditions during production			
No	28	3.68 (1.59)	ref.
Yes	12	5.33 (1.30)	0.001
Inspection time around onset of lay			
< 1 hour per day	25	3.84 (1.60)	ref.
> 1 hour per day	15	4.73 (1.71)	0.085
Inspection time per day later in production			
< 30 minutes per day	11	3.45 (1.63)	ref.
30 - 60 minutes per day	22	4.41 (1.68)	0.097
> 60 minutes per day	7	4.57 (1.62)	0.135
Types of non-edible environmental enrichment			
None	9	3.44 (1.74)	0.053
One type	12	3.92 (1.73)	0.186
≥ 2 types	19	4.68 (1.53)	ref.
Number of inspections after peak of lay[1]			
1 time per day	7	4.14 (1.57)	ref.
2 times per day	25	3.8 (1.68)	0.602
≥ 3 times per day	8	5.38 (1.30)	0.122

792 [1]The variable had only one level with p < 0.2. Log likelihood test (LR chi^2 = 5.82; Prob > chi^2 =
793 0.0546) indicated that the variable should be included in the model prior to backward stepwise
794 reduction.

Table 5. Overview of the explanatory variables with p < 0.2 that were relevant to include in analysis for the flocks housed in furnished cages (FC) (n = 30). Means and standard deviations (SD) are described for each level.

Explanatory variables (FC)	Number of observations	Problem behavior index mean (SD)	P-value
Size of the flock			
< 7,500 birds	6	3.33 (1.03)	ref.
≥ 7,500 birds	24	2.08 (1.32)	0.012
Challenges related to content or distribution of feed			
No	22	2.55 (1.34)	ref.
Yes	8	1.75 (1.28)	0.067
Light intensity adjusted using a LUX-meter			
Yes	21	2.14 (1.31)	0.172
No	9	2.78 (1.40)	ref.
Inspection time around onset of lay			
< 1 hour per day	22	2.5 (1.26)	ref.
≥ 1 hour per day	8	1.88 (1.55)	0.05
Access to environmental enrichment (edible and non-edible) [1]			
Yes	29	2.41 (1.30)	0.044
No	1	1 (-)	ref.

[1] Only one producer did not provide the birds with any sort of environmental enrichment, so this variable had to be excluded from further analysis.

801
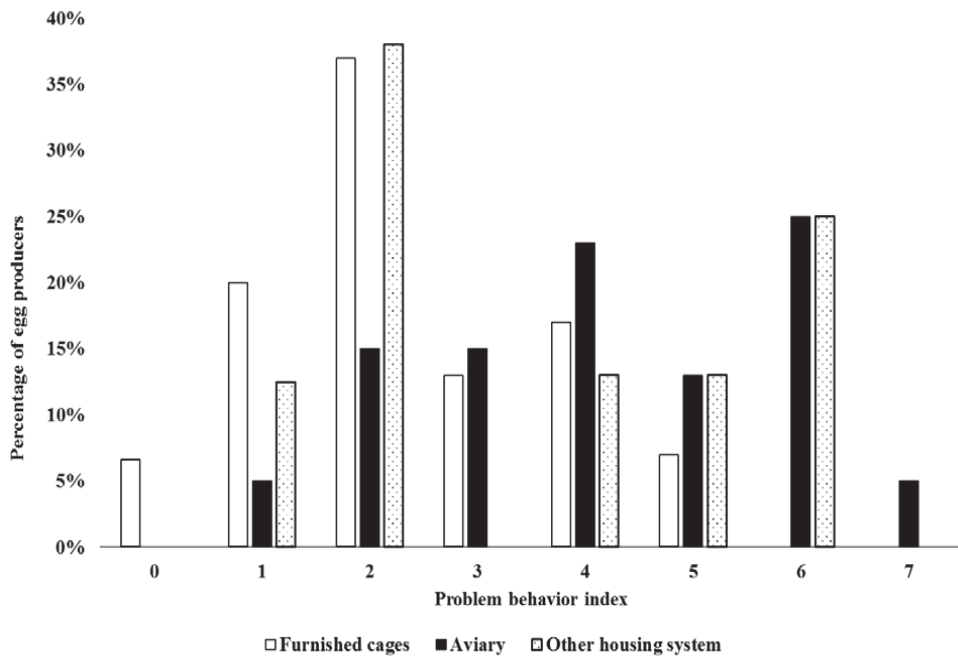
Figure 1: Overview of the percentage of reported problem behaviors grouped by housing system

at the egg production farm (n = 78). Black bars = aviary; white bars = furnished cages; dotted

bars = other housing system (floor systems, aviaries with outdoor access or organic production).

The continuous index ranges from 0 (none of the problem behaviors reported) to 7 (all the

outcomes reported) by the producer.