Effects of adult housing conditions and dark brooder rearing on hippocampal plasticity in laying hens

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Abstract

Hippocampal plasticity, the remodelling of immature neurons in the hippocampus, is downregulated in response to chronic stressors in rodents, especially in the ventral dentate gyrus (DG), while the dorsal DG has a role in spatial memory. A similar functional gradient has been hypothesised along the rostro-caudal axis of the avian hippocampal formation (HF), and previous studies in chickens have found that chronic stress suppresses hippocampal plasticity, especially in the caudal HF. I used hippocampal plasticity to measure the effects of housing systems and related health conditions on chronic stress in laying hens. I also investigated whether dark brooders, which simulate aspects of maternal care during rearing, confer stress resilience to adult hens.

In my first study, I compared hippocampal plasticity between birds which were moved from the same rearing site to either a flat-deck or multi-tier laying system. Brains were stained to visualise doublecortin (DCX), a marker of neural plasticity. There was no difference in DCX^+ cell density in the caudal or rostral HF, suggesting no detectable differences in chronic stress or spatial stimulation. In my second study, I measured DCX^+ cell density in hens with footpad dermatitis (FPD) of varying severity. While hippocampal plasticity tended to decrease in response to FPD in conventionally reared birds, it tended to increase in response to FPD in dark brooder reared birds. Finally, I measured the effects of unpredictable chronic mild stress (UCMS) on behavioural responses, plasma corticosterone, and expression of genes related to hippocampal plasticity in hens reared with or without a dark brooder. In response to UCMS, DCX expression tended to decrease in the caudal HF of conventionally reared birds, but significantly increased in dark brooder reared birds. The results suggest that dark brooders confer stress resilience, and add to the call for their wider implementation on commercial rearing sites.

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AB	Antibody buffer
ACTH	Adrenocorticotropic hormone
AHN	Adult hippocampal neurogenesis
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AVT	Arginine vasotocin
BDNF	Brain-derived neurotrophic factor
BIOM	Biological observation matrix
BNST	Bed nucleus of the stria terminalis
BrdU	Bromodeoxyuridine
CORT	Corticosterone
CRHR2	Corticotropin releasing hormone receptor 2
CSF1R	Colony-stimulating factor 1 receptor
DAB	3,3'-Diaminobenzidine
DCX	Doublecortin
DG	Dentate gyrus
ELISA	Enzyme-linked immunosorbent assay
FPD	Footpad dermatitis
GR	Glucocorticoid receptor
H/L	Heterophil/lymphocyte
HF	Hippocampal formation
HFP	High feather pecking
HPA	Hypothalamic pituitary adrenal

IHC	Immunohistochemistry
KBD	Keel bone damage
LBR	Lamin B receptor
LFP	Low feather pecking
LPAI	Low pathogenic avian influenza
MR	Mineralocorticoid receptor
NOT	Novel object test
OCT	Optimal cutting temperature compound
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCoA	Principle coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
qPCR	Quantitative polymerase chain reaction
SE	Standard error
TI	Tonic immobility
UCMS	Unpredictable chronic mild stress

Chapter 1. General Introduction

1.1. Background

Laying hens (*Gallus gallus domesticus*) are exposed to a wide array of stressors throughout the rearing and production phases, many of which are associated with their housing systems. While there has been much discussion about the benefits of cage-free housing in allowing birds to express natural behaviours by moving around the barn and in some cases accessing an outdoor range, these aspects of cage free housing present novel welfare challenges which are complex and not fully understood. Also, commercial rearing environments often fail to allow expression of natural behaviours in chicks because they cannot access a mother hen. Experiences during early life are known to programme the stress response in mammals(Liu *et al.*, 1997; Nguyen *et al.*, 2015) and birds (Marasco *et al.*, 2016; Walker *et al.*, 2019; De Haas *et al.*, 2021), and therefore it is also important to consider interventions during rearing which may confer long term stress resilience to animals which will be moved into production systems in which stressors are inevitable. Previously, hippocampal neurogenesis has been used to measure long term negative affective states in multiple species, including chickens. In this thesis, hippocampal plasticity is used to investigate the effects of adult housing and related health conditions on chronic stress during adulthood, and whether attempting to simulate maternal care during early life may change how the brain responds to these stressors during adulthood.

1.2. Cage-free laying systems

After conventional cages were banned in the European Union in 2012, farmers using these systems were required to change to alternative systems, such as furnished cages, floor systems, or aviaries. A survey of Belgian farmers (Stadig et al., 2016) found that the most commonly cited reason for choosing a system was consumer demand, closely followed by how easily a system could be implemented on existing farms. More recently, Sinclair et al. (2022) found that 90.2% of consumers in the UK, where the majority of eggs are now produced in cage-free systems, at least somewhat agreed that they would prefer to buy eggs from chickens that have not been housed in cages. Largely due to this popularity with consumers, the egg industry has shifted towards cage-free housing systems, which are generally believed to improve the welfare of laying hens. A variety of cage-free systems are available, and each system may present different issues. It is important to be aware of how these may affect welfare.

In some of these systems, birds are kept indoors but are free to move around the barn. Freedom to move outside of cages allows greater opportunity for exploration and expression of natural behaviours such as dust bathing and nesting (Duncan, 2001). There are a variety of barn designs with differing levels of complexity. The simplest barn systems are flat-deck (or floor) systems, in which birds are housed on a single level on a floor usually with litter. Caged birds often have access to only a mesh floor rather than litter, and have shown high demand for access to litter in order to perform dust bathing and foraging behaviours (Gunnarsson *et al.*, 2000). Though flat-deck systems offer relatively low environmental complexity, enrichment is required in all systems, and structural enrichment perching areas are provided to allow some use of three-dimensional space. Provision of perches has been found to improve bone strength (Lay *et al.*, 2011; Habig and Distl, 2013) and is needed for optimal skeletal development (Campbell, De Haas and Lee, 2019). Providing perches also has behavioural benefits such as reducing fear and improving the transition of pullets from rearing to laying sheds (Campbell, De Haas and Lee, 2019). Some barn designs are more complex, such as multi-tier aviaries which provide more structural enrichment in the form of levels that the birds are free to move between in addition to perches. Therefore, hens in multi-tier aviaries have more opportunity to fulfil natural behavioural needs (Sandilands, Moinard and Sparks, 2009).

Free-range and organic hens have access to further environmental complexity and exploration opportunities through the provision of access to an outdoor range, where they spend most of their time foraging (Campbell, Bari and Rault, 2021). Allowing access to the range allows hens to choose how they interact with their environment, meaning they have more control and can behave according to their needs (Bonnefous *et al.*, 2022), thereby possibly reducing stress.

1.2.1. Disadvantages of cage-free systems

While the perceived welfare benefits of cage-free systems are clearly popular with consumers (Stadig *et al.*, 2016), and the opportunity to move freely within the housing is generally accepted to improve welfare, cage-free housing systems present novel welfare issues for hens which are not yet fully understood. This includes increased prevalence of some health conditions and damaging behaviours.

The selection of modern laying hens for high production means there is a trade-off between calcium deposition in bones and eggshells, which can lead to bone weakness (Jansen *et al.*, 2020). In addition, the anatomy of hens means their keel bone is exposed and only covered with a thin layer of muscle. Therefore, hens are highly prone to damaging this bone. Keel bone

damage (KBD) refers to two main types of injuries: fractures, which are usually detected as calluses indicating older bone breakages (Casey-Trott *et al.*, 2015), and deviations of the keel bone, which may develop over time rather than as the result of a single traumatic injury (Riber, Casey-Trott and Herskin, 2018). Pain from KBD may cause birds to stay on the floor rather than use perches (Nasr, Nicol and Murrell, 2012) and may also reduce the level of production in layers (Nasr *et al.*, 2012) and the quality of eggs (Candelotto *et al.*, 2017). Severe keel bone fractures have been found to cause chronic negative affective states in laying hens (Armstrong *et al.*, 2020a) and result in more fearful behaviours during behavioural tests (Wei *et al.*, 2022).

KBD has been identified as a problem in both caged and cage-free systems (Casey-Trott *et al.*, 2015). The freedom to move around a barn increases the likelihood of collisions with furnishings such as perches and drinkers (Harlander-Matauschek *et al.*, 2015), and provision of structural enrichment is associated with higher prevalence of bone fractures (Sandilands, Moinard and Sparks, 2009). Therefore, movement within cage-free systems has been proposed as a possible risk factor for KBD. In a review of previous studies, Heerkens (2016) reported the prevalence of KBD to be higher in cage-free systems (ranging from 48 – 97% prevalence) compared with conventional cages (ranging from 25 – 42% prevalence). There was a large amount of variation in reported KBD prevalence between studies in furnished cages, ranging from 23% (Habig and Distl, 2013) to 92% prevalence (Hester *et al.*, 2013), which may also indicate that the furnishings can increase the likelihood of injury even in caged birds with less opportunity to move. However, more recently, Thøfner *et al.* (2020) reported a similar range of prevalences of KBD between caged (50 – 98% prevalence) and cage-free systems (53 – 100% prevalence), and they dispute the previous assumptions that external trauma is the primary cause of KBD. Instead, Thøfner *et al.* (2020) propose that the primary cause of KBD may be internal, such as pressure on the caudal tip of the keel bone during egg production, rather than related to the housing environment.

Another common welfare problem in poultry housing is piling, an unpredictable phenomenon in which birds group together in a large mass inside the barn, often resulting in smothering and the death by suffocation of some individuals (Bright and Johnson, 2011). Like KBD, piling can occur in any system, though increased ability to move around the housing means it is much more likely to occur in cage-free systems (Gray *et al.*, 2020). Further study is needed to elucidate the cause of smothering and management practices which could mitigate it.

Another example of a housing-related health condition which will be studied during my project is footpad dermatitis (FPD), in which severely affected individuals have painful necrotic lesions on the feet (Hothersall *et al.*, 2016) which are believed to be mainly caused by prolonged contact with wet bedding (Wang, Ekstrand and Svedberg, 1998). FPD is typically higher in free-range or organic systems due to accessing an outdoor range (in addition to wet bedding inside the barn), which can increase the likelihood of injuries and infections (Pagazaurtundua and Warriss, 2006). Contrary to this, Riber and Hinrichsen (2016) found increased prevalence of footpad lesions in a barn system compared to an organic system, which they suggest may be due to reduced litter quality as a result of high stocking density inside the barn. It appears that keeping birds inside and allowing access to the range both have disadvantages when aiming to avoid high prevalence of FPD.

Usage of the range area is varied in free-range hens. Using RFID tracking, Gebhardt-Henrich, Toscano and Fröhlich (2014) investigated range use in 12 flocks of laying hens. The proportion of hens which visited the range at least once within the first three weeks ranged between 47 and 99%. Factors affecting differences in range use between flocks are mostly environmental differences such as range design and weather conditions (Pettersson, Freire and Nicol, 2016) and flock size, with hens from smaller flocks foraging on the range more frequently and for longer (Gebhardt-Henrich, Toscano and Fröhlich, 2014). Even within flocks there is great variation in range use between individuals, and even when given the opportunity, some individuals choose to never go outside (Campbell *et al.*, 2018). There appears to be variation in cognitive abilities between those that choose to go outside and those that do not (Campbell *et al.*, 2018). It is unclear whether the choice of some individuals to stay inside is a welfare issue, and the reasons for individuals voluntarily staying inside are unknown.

Birds that choose to go outside are at increased risk of predation. Bestman and Bikker-Ouwejan (2020) found that in flocks with an overall mortality rate of 12.2%, wild predators (mainly birds of prey and foxes) were responsible for the deaths of 3.7% of hens. The rate of predation is likely to be underestimated, because mortality due to predation is not always reported by farmers (Bonnefous *et al.*, 2022). It has been suggested that access to the range may also mean that birds are more at risk of pathogenic infections such as low pathogenic avian influenza (LPAI), which can be transmitted from wild birds to domestic poultry. Barnes and Glass (2018) modelled the introduction of LPAI into a variety of commercial poultry systems, and found that free-range access was the greatest factor driving the introduction of

LPAI to flocks. *Salmonella spp.* is an important pathogen in human health, and it has been proposed that providing outdoor access to poultry could increase the risk of *Salmonella* infection, though (Wierup *et al.*, 2017) found no evidence of increased infection rates in free-range hens compared to barn housed hens. Further study is needed to understand other pathogens and welfare issues associated with access to outside.

1.3. Rearing environment

1.3.1. Conventional rearing

Due to the spatial challenges encountered by birds when moving into cage-free laying systems, during the rearing period it is important for pullets to develop behaviours which are appropriate for the laying system to which they will be moved, and physical characteristics such as bone strength which are required for movement around the laying system (Campbell, De Haas and Lee, 2019). There is variation in the levels of complexity in commercial rearing sites. Layer pullets may be reared in furnished cages, or in more complex cage-free systems which provide more opportunities for natural behaviours and therefore promote adaptation to the adult environment (MacLachlan *et al.*, 2020).

1.3.2. Dark brooder rearing

Though conventional rearing sites often use enrichment to improve the welfare of birds early in life and ease their transition to adult systems, they often fail to meet behavioural needs of chicks in other ways. In a natural setting, chicks rely on their mother for thermoregulation during the first weeks of life (Edgar *et al.*, 2016). However, chicks do not have access to their mother on commercial rearing farms because this is not commercially viable. Instead, whole house heating or radiant brooders may be used to provide heat to chicks during early life. Also, chicks are exposed to continuous lighting (Edgar *et al.*, 2016) which may cause poor welfare, and without the presence of a mother hen, they do not have a dark place where they can choose to go to rest.

A dark brooder consists of a heating element surrounded by a dark, enclosed area (Janczak and Riber, 2015). Dark brooders have not been widely implemented in commercial rearing sites (Sirovnik and Riber, 2022), but on some sites, they are used to simulate both the darkness and warmth that would be provided by a broody hen under natural conditions, but are not available to chicks in conventional rearing systems. The duration for which dark brooders are present varies greatly between studies and between systems, from 3 weeks in fast growing broilers in experimental pens (Forslind *et al.*, 2022) up to 16 weeks in laying chicks on a

commercial farm (Gilani, Knowles and Nicol, 2012). Chicks tend to use dark brooders less after the first week of life, but they are typically kept available for longer, because in addition to providing welfare benefits they are more economical than whole-house heating (Sirovnik and Riber, 2022).

Welfare benefits of dark brooders have been studied in both broilers and laying hens. Broilers show longer bouts of resting and are less likely to be disturbed during resting bouts if they have access to dark brooders (Forslind *et al.*, 2022). These effects continued after the brooders were removed. Laying hens reared with a dark brooder also rested more compared to those reared in barren pens (Campderrich *et al.*, 2019). Though, in that study, dark brooders were treated as one of many structural enrichment items intended to add complexity to the environment, so it is not clear whether the improvements in welfare were due to simulated maternal care or a more general increase in environmental complexity.

Previous studies investigating the benefits of dark brooders on laying hen welfare have largely focused on dark brooders as an intervention to reduce injurious pecking (reviewed by Sirovnik and Riber, 2022), which is a common problem in poultry which is associated with chronic stress and the housing environment (El-Lethey *et al.*, 2000; Rodenburg *et al.*, 2013). The long term positive effects of dark brooders on injurious pecking was first reported by Jensen, Palme and Forkman (2006), who found that severe feather pecking was completely absent in layer pullets reared with a dark brooder, but was highly prevalent in pullets reared with heat lamps, as is more common commercially. Subsequent studies have confirmed that dark brooders reduce the prevalence of injurious pecking (Gilani, Knowles and Nicol, 2012; Riber and Guzman, 2017). Hens reared with a dark brooder also use nests more readily, leading to fewer floor eggs and also have reduced mortality compared to hens reared without brooders (Riber and Guzman, 2017).

In studies in mammals, maternal care early in life appears play a role to programming the stress response, with effects lasting into adulthood (Liu *et al.*, 1997; Champagne *et al.*, 2008; Nguyen *et al.*, 2015; Nguyen *et al.*, 2018). The welfare benefits of dark brooders observed in previous studies suggest that simulating maternal care in chicks may have a similar effect in conferring resilience to stressors during adulthood, including through programming of the stress response during early life (De Haas *et al.*, 2021) though further study is needed to understand the mechanisms by which dark brooders may confer stress resilience.

1.4. Measures of laying hen welfare

1.4.1. Behavioural measures

When aiming to reduce stress and improve animal welfare, refinement of housing systems and husbandry procedures are both needed. In order to do these effectively, we must be able to understand and accurately quantify stress. Behavioural tests are important when investigating which aspects of housing systems affect welfare, because they allow us to observe what animals choose to do in a given environment (Broom, 1986). Previously, stress and anxiety have been studied in chickens in behavioural studies, using a range of validated behavioural tests (Forkman *et al.*, 2007). In some behavioural tests, animals are placed in an environment which may be stressful, or at least unfamiliar, and multiple defined behavioural responses are recorded as welfare indicators. The purpose of measuring these welfare indicators is to quantify the effort of an individual to cope with the stressful environment (Broom, 1986).

Tonic immobility (TI) is one of the most common fear tests used in hens (Forkman *et al.*, 2007). In this test, a bird is restrained on its back until tonic immobility is induced, meaning the bird lies still even after restraint is removed. The duration of TI is recorded as the time between restraint being removed and the bird righting itself, and this is expected to be longer in more fearful animals (Jones and Waddington, 1992). Increased TI times have been observed in chickens following a variety of acute stressors including rough handling (Jones, 1992) and restraint in a crush cage (Marin *et al.*, 2001). Hens housed in conventional cages were found to have increased TI duration compared to those housed in floor pens, suggesting that chronic stress caused by the housing environment also increases TI duration (Campbell *et al.*, 2022), however, Gualtieri *et al.* (2019) did not find a significant effect of unpredictable chronic mild stress (UCMS) on TI duration. There is also an effect of strain on TI, with longer durations recorded in white hens compared to brown hens (Fraisse and Cockrem, 2006). TI duration has been decreased by housing birds with enrichment (Jones and Waddington, 1992).

Another common way to test behavioural welfare indicators is to place individual birds in a novel environment, most commonly in an open field test (Forkman *et al.*, 2007). Behaviours during an open field test are best explained by the need to be reunited with conspecifics and avoiding the possibility of predation (Gallup and Suarez, 1980; Suarez and Gallup, 1983), which is understandable after an individual bird has been captured by a human and separated from its conspecifics. Open field tests may also be used to test fear or anxiety (as in Nordquist *et*

al., 2011), and allow for the recording of a wide range of behaviours. Testing in an open field may be conducted with or without food, and latency to eat may be a useful measure of anxiety (Campbell, Dickson and Lee, 2019).

Rather than placing birds in an unfamiliar open field, they may be placed in a familiar arena with a novel object. In addition to behaviours such as freezing and movement that may be measured in an open field test, a novel object test (NOT) also tests willingness to interact with a novel object as a measure of anxiety. This may be conducted with individuals in a testing arena (Fraisse and Cockrem, 2006; Wei *et al.*, 2022) or with a large group of individuals in the home pen, making it an easy test to conduct (Forkman *et al.*, 2007). Fraisse and Cockrem (2006) found no differences in behaviours between strains during a novel object test, even though they did find a difference in TI in the same birds. A single behavioural test may not accurately measure fear or anxiety in hens due to different motivations for behaviours, and Campbell, Dickson and Lee (2019) suggest that multiple behavioural tests should be conducted with hens.

Another type of behavioural test which is commonly used to investigate welfare involves training birds to associate events with stimuli. For example, Zimmerman *et al.* (2011) trained laying hens to associate auditory stimuli with either a positive or negative experience. Baseline behaviours were recorded during a waiting period, then an auditory stimulus was presented and the behaviours expressed by individuals within 15 seconds after hearing the stimuli were recorded. After a positive stimulus, birds tended to express more behaviours which were broadly categorised as "comfort" behaviours (including preening, feather ruffling and tail wagging) compared to baseline, while birds presented with a negative stimulus expressed significantly fewer comfort behaviours. This suggests that birds can anticipate both positive and negative experiences based on stimuli, and therefore similar tests can be used to investigate underlying cognitive biases of animals from different conditions.

In broiler chicks, the complexity of the rearing environment did not affect results of a judgement bias test or duration of tonic immobility (Lourenço-Silva *et al.*, 2023). However, laying hen chicks are likely to interact with the rearing environment differently so these findings in broilers may not be generalisable to laying hens. At five weeks of age (and after an unpredictable stress protocol), laying hens reared in less complex environment had greater increase in negative judgement bias from a baseline judgement bias test conducted at two weeks of age (Zidar *et al.*, 2018).

An alternative to conducting behavioural tests is observing the instances of behaviours that are believed to be associated with stress. One such behaviour which is commonly studied in chickens is feather pecking (El-Lethey *et al.*, 2000; Rodenburg *et al.*, 2013). Feather score, or body condition more generally, may also be used as an indicator of welfare at flock level.

1.4.2. Physiological measures

The Hypothalamic pituitary adrenal (HPA) axis is a major component of the stress response, which is conserved across species and is involved in the regulation of many physiological processes (Sheng *et al.*, 2021). The main role of the HPA axis is to regulate the release of glucocorticoid hormones, specifically corticosterone (CORT) in birds, from the adrenal gland (Smulders, 2021). Stress may be quantified in laying hens by directly measuring the output of the HPA using CORT titres. CORT is measurable in blood plasma, saliva, and feathers (Nordquist *et al.*, 2020) and is elevated following acute stressors. Chronic stimulation of the HPA axis may reduce baseline CORT titres (Gualtieri *et al.*, 2019).

The immune system is known to be sensitive to stress, and therefore immune markers are commonly used to assess stress in hens. For example, stressful housing conditions have been found to affect heterophil/lymphocyte (H/L) ratio in the blood (El-Lethey *et al.*, 2000), though Gualtieri *et al.* (2019) did not find a significant effect of UCMS on H/L ratio. Also, following an "intermittent stressful challenges protocol" (an array of stressors at unpredictable times for 5 days, including changes to temperature, unpredictable lighting, and unfamiliar loud noises), birds reared in complex pens (including a dark brooder) were found to have lower H/L ratios than those reared in barren pens (Campderrich *et al.*, 2019). This suggests that dark brooders may reduce stress, which is consistent with the welfare benefits of dark brooder rearing discussed earlier.

Other immune markers that are commonly measured include CD4+ and CD8+ T cells. Matur *et al.* (2015) found increased proportions of CD4+ and CD8+ cells in the blood of 17-week old hens after exposure to social stress. This was observed in birds reared in furnished cages, but not those reared in conventional cages, suggesting the rearing environment influences the effects of social stress on the immune system.

1.5. Hippocampal structure and function

1.5.1. Mammalian HF

The mammalian hippocampal formation (HF) is a large brain structure which is located deep within the medial temporal lobe of humans, and lies below the neocortex in rodents (Knierim, 2015). The HF consists of the dentate gyrus (DG), *Cornu ammonis* (or Ammon's horn, consisting of CA fields CA1-CA4), subiculum, and entorhinal cortex (Gupta *et al.*, 2012). CA3 is the largest of the subfields of the *Cornu ammonis*, and receives input from granule cells in the DG via mossy fibres (Viana da Silva *et al.*, 2019). The mammalian HF has a number of roles involved in memory, spatial navigation, and emotional behaviour (Anand and Dhikav, 2012). Following the well-known study by Scoville and Milner (1957) in which the patient known as "H.M" lost the ability to form new memories after a large portion of his hippocampal formation (HF) was removed, studies concerning the function of the HF have mostly focused on learning and memory. However, more recently, more studies have investigated the role of the HF in emotional behaviour and mood disorders such as major depressive disorder (Campbell and Macqueen, 2004; Dranovsky and Hen, 2006). There is a functional gradient along the septo-temporal axis or (dorso-ventral axis in rodents) of the mammalian hippocampus (Moser and Moser, 1998). Many studies have shown that lesions in the dorsal region of the hippocampus impair spatial memory, while performance in spatial memory tasks is not significantly affected by lesions in the ventral DG (Bannerman *et al.*, 2014; Bannerman *et al.*, 2004). This supports the hypothesis that the ventral pole of the HF has a separate function to the dorsal pole.

The HF is also involved in negative feedback in the hypothalamic-pituitary-adrenal (HPA) axis (Jacobson and Sapolsky, 1991), an important component of the stress response, specifically through the expression of glucocorticoid receptors (GR). Neurons in the temporal pole of the hippocampus project to the HPA axis and amygdala (Bannerman *et al.*, 2014). Kheirbek *et al.* (2013) used optogenetics to activate granule cells in specific regions of the DG in mice, and found that activating granule cells in the ventral DG suppressed innate anxiety behaviours, rather than affecting contextual learning and exploration as was observed when cells in the dorsal DG were activated. (Anacker *et al.*, 2018) demonstrated that neurogenesis in the ventral DG plays a role in inhibiting the activity of mature granule cells, allowing greater resilience to chronic stress.

1.5.2. Avian HF

The avian HF is located in the dorsomedial region of the teIencephalon (Atoji, Sarkar and Wild, 2016). In order for hippocampal plasticity to be used as a valid measurement of chronic stress in other species, the HF must play a similar role in the mediation of stress and anxiety as in humans and rodent models that have been studied previously. There are great morphological differences between the HF of mammals and birds, however, it has been proposed that there are functional similarities (Gupta *et al.*, 2012). Herold *et al.* (2014) argue that although there appear to be many functional similarities between the mammalian and avian HF, the great structural and organisational differences resulting from 300 million years of independent evolution may mean it is unhelpful to our understanding of the avian HF to think of it in mammalian terms, and they suggest that further study is required to elucidate its function. However, Atoji, Sarkar and Wild (2016) suggest that patterns of the expression of genes such as Prox1 provide evidence that the V-shaped region of the avian hippocampus is homologous to the mammalian DG.

Much like the mammalian HF, The avian HF is in involved in regulation of the stress response, and contains a high density of glucocorticoid (MR) and glucocorticoid receptors (GR) (Dickens *et al.*, 2009) which are necessary for control of the HPA axis by negative feedback. Bouillé and Baylé (1973) stimulated multiple sites along the HF of pigeons, and found that CORT titres were most strongly suppressed when the ventral HF was stimulated at the most caudal site. This suggests that the caudal pole has a different function to more rostral sites in the HF.

There is evidence of functional specialisation along the rostro-caudal axis of the avian HF, and it has been proposed that there is a similar functional gradient to that observed in mammals (Smulders, 2017). The rostral HF is homologous to the mammalian dorsal hippocampus, with a role in spatial cognition and memory, and is the site of the majority of place cells in the avian HF (Payne, Lynch and Aronov, 2021). The caudal HF is sensitive to emotional stimuli and plays a role in the stress response (Smulders, 2017). However, there is some evidence against the hypothesised functional gradient of the avian HF. There are projections to the bed nucleus of the stria terminalis (BNST) along the avian HF (Atoji, Saito and Wild, 2006), though there is no evidence that there is rostro-caudal specialisation of these projections.

1.6. Hippocampal plasticity

When studying the effects of potential chronic stressors which animals are exposed to throughout their lives, validated measures of cumulative experience are needed (Poirier *et al.*,

2019). Hippocampal plasticity is one such marker of chronic stress. Hippocampal plasticity refers to the addition, growth, migration, or remodelling of neurons as they develop and become integrated into hippocampal networks. Hippocampal plasticity has been used as a marker of cumulative experience in mammals and birds, and is an appropriate measurement of stress in this thesis, because it is an indicator of chronic stress rather than transient affective states, and is therefore more likely to detect meaningful differences in the cumulative experiences of hens between housing systems than behavioural methods or physiological methods of acute stress alone.

1.6.1. Adult hippocampal neurogenesis

Adult hippocampal neurogenesis (AHN) is an extreme form of hippocampal plasticity in which newborn neurons are added to the hippocampus. It was previously believed that newborn neurons only arise in the brain during embryonic development, until Altman (1962) found the first evidence of AHN in the mammalian brain. After intercranial injection of [^{3}H]-thymidine (a marker of cell proliferation), some neurons in the hippocampus of adult rats were labelled with this marker, meaning they had proliferated recently. The granule cell layer (GCL) of the DG is the site of AHN in mammals (van Praag *et al.*, 2002). As these newborn cells mature, projections migrate from the GCL throughout the hippocampal network and may form synapses with pyramidal cells of the CA3 region within one week of their terminal mitosis (Ide *et al.*, 2008).

Eriksson *et al.* (1998) first demonstrated AHN in the human brain. There has since been much debate about how long into adulthood new neurons continue to be added to the human hippocampus, with some arguing that AHN declines shortly after childhood (Sorrells *et al.*, 2018), and others claiming to have found new-born neurons being generated throughout adulthood, with one patient still showing signs of AHN at 79 years of age (Boldrini *et al.*, 2018). Regardless of the age at which AHN begins to decline in human patients, it is known to be downregulated in those who suffer from major depressive disorder (Samuels and Hen, 2011) and other or long term negative affective states such as chronic stress (Dranovsky and Hen, 2006).

AHN has been impaired in mice in response to depression-like states, including those induced experimentally by chronic CORT administration (David *et al.*, 2009) and chronic pain induced by partial nerve ligation (Dimitrov *et al.*, 2014). It has also been demonstrated that the decreased AHN induced in mouse models of depression can be reversed by chronic treatment

with antidepressant drugs such as fluoxetine (Holick *et al.*, 2008). Given our knowledge of the relationship between neurogenesis and depression in humans and rodents, hippocampal plasticity may be a useful tool to quantify stress and depression-like states in other species who cannot verbally self-report the state of their mental wellbeing.

1.6.2. Development and migration of immature neurons

As previously stated, the addition of newborn neurons to the hippocampus is an extreme form of hippocampal plasticity, and is not the only kind of neural plasticity which occours in the hippocampus during adulthood. Hippocampal plasticity also refers to measurement of the density of immature cells, though not necessarily newborn cells (Piumatti *et al.*, 2018), which are still migrating and becoming integrated into hippocampal networks. In addition to measuring plasticity by the density of immature neurons in the HF, the changing morphology of immature cells may also indicate plasticity. When becoming integrated into networks, immature cells may change from a fusiform shape to a rounder shape with more dendritic processes (Boseret, Ball and Balthazart, 2007). UCMS has been found to affect hippocampal plasticity in chicks, measured by changes in dendritic branching and axonal length (Arya *et al.*, 2024).

1.6.3. Neural markers of hippocampal plasticity

A common method of quantifying hippocampal plasticity is to count cells expressing specific molecular targets which are only present in actively dividing cells. These can be exogenous markers, which are labelled compounds that are administered to animals then incorporated into dividing cells, or endogenous markers, which are naturally occurring molecules which have a role in cell division or plasticity and are therefore only expressed in immature cells.

In the early study on AHN in rats, Altman (1962) administered [^{3}H]-thymidine, an exogenous radiolabelled nucleoside which is incorporated into DNA during the S phase of mitosis (Barnea and Nottebohm, 1994). [^{3}H]-thymidine has also been used to quantify hippocampal plasticity in black capped chickadees (Barnea and Nottebohm, 1994). Any cells in which [^{3}H]-thymidine is detected in the nucleus must have divided after the injection, which allows accurate quantification of the number of cells dividing within a known time frame. However, Hu *et al.* (2002) advises against the use of [^{3}H]-thymidine because it can interfere with DNA synthesis and cell morphology. More recently, bromodeoxyuridine (BrdU) has been preferred as an exogenous marker of hippocampal plasticity. BrdU is an analogue of thymidine which has the benefit of detectability using immunohistochemical methods (Duque and Rakic, 2011).

Eriksson et al. (1998) used BrdU to demonstrate AHN in humans using post mortem tissue from cancer patients which were injected with BrdU for diagnostic purposes. BrdU has also been used to quantify AHN in chickens (Robertson *et al.*, 2017).

In studies using a small sample of animals, exogenous markers can be easily administered to all animals and may be preferred to endogenous markers because they provide more information about the time at which cells were generated. However, when sampling a small number of animals from a large flock (as in this some studies in this thesis), endogenous markers are more convenient because they eliminate the need to administer the marker then recapture focal birds. Like BrdU, endogenous markers may be quantified using immunohistochemical methods. In addition, endogenous markers have the advantage of quantification using gene expression which is less time consuming than counting cells.

Doublecortin (DCX) is an endogenous marker of neural remodelling. It is a microtubule binding protein that is involved in the migration of immature neurons (Brown *et al.*, 2003) during their integration into the hippocampal network and it has been argued that it is more accurate than exogenous markers such as BrdU, which only identifies new-born neurons at the time of administration rather than those formed in the previous weeks (Couillard-Despres *et al.*, 2005). DCX is a valuable endogenous marker of hippocampal plasticity in birds (Balthazart and Ball, 2014). In male canaries, high densities of DCX^+ neurons have been found in the nidopallium and HF, which are regions where new neurons are known to be added to the avian brain during adulthood (Boseret, Ball and Balthazart, 2007).

One potential problem with the use of DCX as a marker of neurogenesis was identified by Kremer *et al.* (2013), who found evidence for a pool of "non-neurogenic DCX-protein" which could skew the results of neurogenesis studies. However, by their own admission, this non-neurogenic DCX is at such a low concentration that it is unlikely to be detectable in immunohistochemical studies.

While used as a marker of AHN specifically in some studies, it has been suggested that DCX cells are not necessarily newborn. Piumatti *et al.* (2018) injected sheep with BrdU in order to identify the time at which neurons were generated. They found DCX^+ cells in the cortex of adult sheep which were generated during embryogenesis, not during adulthood as is often assumed of DCX^+ cells. Though the cells are not "immature" in the sense that they are newborn, they appear as immature cells into adulthood (La Rosa *et al.*, 2020), and later develop and become integrated into neural networks.

DCX is the main marker used in experimental chapters of this thesis, and for the reasons stated above, term "hippocampal plasticity" is preferred throughout the experimental chapters of this thesis rather than AHN. Though the age of DCX^+ cells is uncertain, DCX has been used to quantify chronic negative welfare states in previous studies in mice (Terada *et al.*, 2008; Dimitrov *et al.*, 2014; Gualtieri *et al.*, 2017) and chickens (Gualtieri *et al.*, 2019; Armstrong *et al.*, 2020a; Armstrong *et al.*, 2020b; Armstrong *et al.*, 2022). Therefore, quantification of DCX is a validated method for measuring chronic stress in the following experimental chapters.

Brain-derived neurotrophic factor (BDNF) is another endogenous marker of neural plasticity which is highly expressed in hippocampal neurons (Hofer *et al.*, 1990). BDNF promotes dendritic growth and changes in morphology of immature neurons (Kellner *et al.*, 2014) and is essential for neuronal differentiation and survival (Miranda *et al.*, 2019). BDNF is involved in the mediation of neural changes related to learning and memory in the healthy brain (Miranda *et al.*, 2019) and has a critical role in the mediation of mood disorders such as depression (Yang *et al.*, 2020). In addition to its extensive use as a marker of neuronal development in mammalian studies, BDNF has been used to study the effects of providing perches in the rearing system on the brain of layer pullets, though provision of perches was not found to affect dendritic morphology or BDNF expression in the HF (Pullin *et al.*, 2022).

Proliferating cell nuclear antigen (PCNA) is expressed throughout the cell cycle in dividing cells (Zhang and Jiao, 2015), and is not specific to neurons. PCNA is essential in DNA replication due to its role as a processivity factor for DNA polymerase (Kelman, 1997). Though its role is not specific to neural development, PCNA expression can be used to quantify cell division more broadly in the hippocampus as an indicator of neural plasticity.

1.6.4. Hippocampal plasticity as a measure of chronic stress in chickens

Recent studies in chickens have found that plasticity in the avian hippocampus is reduced by a variety of stressors, especially in the caudal HF. Robertson *et al.* (2017) investigated the effect of feed restriction on AHN in broiler breeders by measuring the density of $BrdU^+$ cells in the HF. As these birds have a high motivation to eat, feed restriction was a chronic stressor. They found that feed restricted birds produced fewer new neurons in the rostral HF than those with ad libitum access to food, however the expected difference in AHN at the caudal pole was not detected between these two groups. They did, however, find that reduced AHN was associated with a marked increase in CORT, which has been used as an indicator of stress in

many other studies. This strengthens the evidence that the avian HF responds to chronic stress in a similar manner to the mammalian HF.

Gualtieri *et al.* (2019) measured the density of DCX^+ neurons in the HF to compare hippocampal plasticity between chickens which had been exposed to an unpredictable chronic mild stress (UCMS) paradigm and controls. In this study, the expected effect of hippocampal subregion was observed, with birds exposed to 8 weeks of UCMS having significantly fewer newborn neurons in the caudal HF than controls. More recently, Golgi-Cox staining has been used to visualise neuronal remodelling in response to UCMS (Arya *et al.*, 2024; Tamta *et al.*, 2023). Reduced DCX^+ cell density has been found throughout the HF of hens which had detectable keel bone fractures 3-4 weeks before their brains were sampled (Armstrong *et al.*, 2020a). They also found that hens with fractures acquired earlier in life had a lower density of immature neurons at the caudal pole of the HF than birds with more recent first fractures. Poor body condition has also been found to decrease the density of DCX^+ neurons in the avian HF (Armstrong *et al.*, 2022), though in that study there was no difference in hippocampal plasticity between hens housed in multi-tier aviaries compared to those housed in enriched cages. This suggests that the design of the housing may be a more subtle stressor compared to body condition, KBD, and experimentally induced UCMS. This thesis will further investigate putative stressors which may be more subtle than those investigated previously.

1.7. Aims and hypotheses

In summary, the existing literature suggests that cage-free housing systems that are used during the rearing and laying periods present novel challenges to hens which can cause welfare issues throughout life. The interactions between these challenges are complex, and require well validated biomarkers of cumulative experience in order to fully understand their effects on welfare, and aid in the development of early life interventions which may moderate stress later in life. Previous studies have shown promising results using hippocampal plasticity to quantify cumulative experience in hens, though further research is needed to understand how the brain is affected by more subtle aspects of adult housing systems and their interactions with early life experiences.

1.7.1. Aims

In this thesis, I will use hippocampal plasticity as a marker of chronic stress in housing-related conditions which are more subtle than those investigated previously. Specifically, I will quantify chronic stress in hens which were reared under the same conditions then moved to

two laying systems of differing complexity (Chapter 2). In doing so, I aim to investigate whether this difference in complexity causes chronic stress. I also aim to evaluate the efficacy of hippocampal plasticity of a biomarker of more subtle stressors in laying hens.

In addition to stress caused by the design of the housing directly, housing systems can increase the likelihood of some health conditions which may be stressful. Therefore, in my second study, I aim to use hippocampal plasticity to measure the response to FPD of varying severity.

Finally, I aim to investigate the effects of dark brooders on resilience to stress during adulthood. This aim will be addressed in both my second and third studies. In my second study (Chapter 3), I will investigate whether the effect of FPD on hippocampal plasticity differs between birds reared conventionally or with a dark brooder. In my third study (Chapter 4), I will quantify hippocampal plasticity and plasma CORT in response to experimentally induced chronic and acute stress in birds reared conventionally or with a dark brooder.

1.7.2. Hypotheses

In order to achieve the aims above, I will test the following hypotheses in this thesis. Firstly, I hypothesise that hens will experience higher spatial stimulation and lower levels of chronic stress when moved to a higher complexity laying system compared to hens which are moved to a lower complexity laying system from the same rearing site.

Secondly, I hypothesise that FPD will cause chronic stress. I predict that this will both decrease hippocampal plasticity at the caudal pole of the HF, and will change the composition of the caecal microbiome.

Finally, I hypothesise that rearing chicks with a dark brooder confers resilience to stress during adulthood. I predict that the effects of both FPD and experimentally induced chronic stress on hippocampal plasticity will be smaller in adult hens reared with a dark brooder compared to those reared conventionally.

Chapter 2. Does complexity of aviary design affect hippocampal plasticity in laying hens?

2.1. Abstract

Laying hens are housed many different systems during the rearing and production periods, with cage-free systems such as flat-deck (or floor) housing and multi-tier aviaries increasing in popularity in recent years. While cage-free systems are generally considered to provide better welfare for hens than caged systems due to greater opportunity to express natural behaviours, they present an array of novel welfare challenges that are not yet fully understood. This study compared two cage-free housing designs with different levels of environmental complexity. I hypothesised that hens housed in a more complex aviary experience higher spatial stimulation than those housed in a less complex system, in which they have greater opportunity to explore, and that hens housed in a more complex aviary experience lower levels of chronic stress than those housed in a less complex system.

Pullets were sampled from a flat-deck rearing site at 14 weeks of age. Two weeks later, some of the remaining birds were moved into flat-deck adult housing, and the other remaining birds were moved into a multi-tier aviary. At 24 weeks of age, 12 birds were selected from each group. Brains were collected from all three groups (pullets, flat-deck, multi-tier). I counted cells in the hippocampal formation (HF) expressing doublecortin (DCX), a marker of neural plasticity, which has previously been found to decrease in the avian caudal HF in response to chronic stressors. I expected that DCX^+ cell density in the rostral HF, which has a role in spatial memory, would be significantly higher in the multi-tier group because of greater spatial stimulation. There were no significant differences in DCX^+ cell density among the housing groups. The difference in environmental complexity was therefore not great enough to have a detectable effect on hippocampal plasticity, suggesting that any welfare differences between the systems would be minimal.

2.2. Introduction

As discussed in Chapter 1, laying hens are housed in systems which vary greatly in environmental complexity. Differences in complexity between the rearing site and laying system may introduce spatial challenges for hens, but also greater opportunity to explore and express natural behaviours. Here, I investigate the effects of the transition from a rearing site

to two laying systems of differing environmental complexity on spatial stimulation and chronic stress.

2.2.1. Effects of environmental complexity in rodents

Many studies on the effects of environmental complexity have been conducted in laboratory rodents. Ferchmin and Eterović (1977) trained rats to complete a task in which they were required to walk along a wire ceiling and through barriers to gain a food reward. Individuals that were housed in cages with toys as enrichment were able to first succeed at this task sooner than individuals which were housed singly in barren cages, suggesting an effect of environmental complexity on the development of motor skills.

Environmental complexity has been shown to affect the morphology of the mammalian hippocampus, which is sensitive to environmental stressors. For example, the medial hippocampus was significantly larger in rats housed in an enriched environment than in litter mates paired by body mass which were housed in a barren environment (Walsh *et al.*, 1969). In another study by Fiala, Joyce and Greenough (1978), rats were housed either singly in barren cages, or in groups of 6-12 rats in larger cages with a selection of toys which were changed daily, then brains were collected and Golgi-stained to visualise dendritic branching. Compared to the isolated rats, higher dendritic branching was observed in granule cells in the dentate gyrus of the hippocampus of rats in the enriched cages 4 weeks after weaning, but there was no effect of environmental complexity on dendritic branching 12 weeks after weaning. This suggests that neural development in the hippocampus is particularly sensitive to environmental complexity in early life. Contrary to these studies, Grégoire *et al.* (2014) found that increased environmental complexity (presence of coloured plastic tunnels which were regularly rearranged, and a locked running wheel) did not affect hippocampal neurogenesis in rats, however, voluntary running did increase neurogenesis in rats which had access to a working running wheel but an otherwise low-complexity environment with no tunnels. Rats housed in the complex environment for 4 weeks did have reduced baseline blood plasma corticosterone compared to control rats from an empty environment. This reduction in corticosterone was not observed in rats with the running wheel, suggesting that the reduced baseline stress was a result of environmental complexity, rather than exercise alone.

In mammals, there is a functional gradient along the dorso-ventral axis of the dentate gyrus (DG). The dorsal DG has a role in spatial memory, while the ventral DG is sensitive to emotional stimuli and is associated with responses to stress and anxiety (Bannerman *et al.*, 2004).

Gualtieri *et al.* (2017) investigated the effects of environmental enrichment on the expression two markers of neural plasticity (DCX and BDNF) in the DG. At 11 weeks of age, female mice were housed in standard cages for 12 days, then allocated to either an enriched cage (a complex environment in a larger cage containing plastic tubes, igloos, running wheels, and urine from male mice) or a control cage (a standard sized cage with no enrichment and minimal complexity) for 8 days. BDNF and DCX were quantified in the DG by immunohistochemical staining and mRNA expression. There was no effect of environmental complexity on BDNF immunoreactivity or gene expression, however there was significantly higher BDNF immunoreactivity and gene expression in the ventral DG than the dorsal DG. There was an interaction between enrichment and region of the DG on DCX, with higher DCX immunoreactivity in the dorsal DG of mice from the enriched environment compared to control mice, but lower DCX immunoreactivity in the ventral DG of enriched mice. For enriched mice, DCX gene expression was higher in the dorsal DG, but lower in the ventral DG compared to controls. This was unexpected because decreased DCX expression in the ventral DG is believed to indicate chronic stress, which was expected to be observed in the less complex environment rather than the more complex environment.

2.2.2. Effect of environmental complexity in laying hens

Previous studies have investigated the effects of environmental complexity on various welfare measures in laying hens. Armstrong *et al.* (2022) compared the density of DCX^+ cells in the hippocampal formation (HF) of laying hens with either good or poor body condition, which were housed in either enriched cages or a free-range multi-tier aviary, in which the birds had much more freedom to explore the environment and perform natural behaviours. Across the whole HF, there was no significant difference in bipolar or multipolar DCX^+ cell density between the caged and free-range birds, and no interaction between housing condition and body condition on DCX^+ cell density. The results from Armstrong *et al.* (2022) suggest that there was no detectable difference in chronic stress or spatial stimulation between hens housed in these two systems. While all birds used in that study were reared in similar floor systems managed by the same pullet rearing company, each housing system contained a different strain of hens (Hy-Line in enriched cages, H&N in multi-tier aviary), so the groups were not reared together, and housing system was confounded by strain. It is possible that different strains of hens will have different responses to environmental complexity, so some strains may be more suited to a particular housing type than other strains.

In a study by Ross *et al.* (2020), laying hens were housed in 1.5m^2 pens with a single perch and nest box, while other hens were housed in larger enriched pens (9m^2), including regularly replaced enrichment items (grass, dust for bathing) and permanent enriching features such as raised platforms and perching areas at a variety of heights. This study was conducted in two cohorts (summer and autumn), with six groups of four hens from each cohort housed in each condition. After 5-6 weeks in the experimental pens, startle responses were tested by measuring force exerted against a force plate in response to a bright flashing light in a dark chamber. Hens from the enriched environment had significantly reduced startle responses compared to the birds in the less complex control environment. Because the enriched pens were much larger than control pens, complexity may have been confounded by floor area or stocking density in this study.

A subset of the hens studied in Ross *et al.* (2020) were assessed for baseline exploratory behaviour, and classified as either exploratory or non-exploratory hens (Ross *et al.*, 2019). These individuals were then trained to distinguish between cues associated with positive and negative events, and were exposed to a judgement bias test in which their responses to an ambiguous stimulus were measured. Exploratory birds housed in an enriched environment showed more optimistic behaviour than exploratory birds from control environment, while there was no effect of environmental enrichment in the non-exploratory birds. This suggests that individual personality may be an important factor in responses to environmental complexity.

Commercially, further complexity may be added to the environment by using aviary systems, in which multiple levels are permanently available to the birds, allowing more use of the vertical space inside the barn. This also allows more efficient use of the floor area, and can house more animals in same space as a flat-deck system. While higher complexity cage-free systems such as multi-tier aviaries allow greater opportunity for birds to explore and express natural behaviours, they have some significant disadvantages such as increased chance of injuries, especially keel bone fractures (Sandilands, Moinard and Sparks, 2009). More complex systems also have disadvantages for ease of management of hygiene, as it is more difficult to maintain the quality of the litter than in caged systems, where birds are separated from the floor level (Duncan, 2001).

While some cage-free systems are indoor only, free-range systems in which birds have access to an outdoor range are also very popular. Access to an outdoor range presents further

welfare challenges such as increased disease susceptibility and possibility of predation (Campbell, Bari and Rault, 2021), though these risks are considered to be outweighed by welfare benefits such as the freedom to explore and perform more natural behaviours. There is variation in range use between individual hens, and some choose not to go outside even when given the opportunity to do so. Campbell *et al.* (2018) found that birds who never use the outdoor range were the slowest to achieve success in a T-maze learning task. Also, birds reared in an enriched environment with a lower latency to success in the T-maze learning task were more likely to use the range, though this effect was not observed in the birds reared in a lower-complexity control environment. This suggests that range use is related to cognitive abilities, which may be influenced by the rearing environment.

2.2.3. Changes in complexity from the rearing site

There is also variation in the levels of complexity in the rearing site. Rearing at floor level or in a single or double tiered aviary did not affect BDNF gene expression or hippocampal dendritic morphology at 16 weeks of age (Pullin *et al.*, 2022), suggesting the difference in complexity between these rearing systems was not great enough to affect the brain. Changes in environmental complexity commonly occur in laying hens as they are moved from the rearing site to an adult laying system, and this movement to a system with great difference in complexity may cause fear or stress, and consequently, injurious behaviours such as feather pecking (Janczak and Riber, 2015). Environmental complexity during rearing may affect the way birds adapt to an adult environment, both physically and cognitively, which may have implications for welfare.

Aviary-reared birds maintained bone strength best when moved to aviaries for laying, compared to birds moved from aviaries to conventional cages which had decreased bone strength (Regmi *et al.*, 2016). This may have been due to reduced opportunity for locomotion, meaning the bone strength developed in a complex rearing environment could not be maintained. In a review of studies investigating the effects of enrichment on the development of laying hen pullets, Campbell, De Haas and Lee (2019) suggest that with the increasing popularity of more complex laying systems such as multi-tier aviaries, rearing in similar systems is increasingly important for physically preparing birds for movements such as jumping and flying that are necessary for navigation of such environments. The complexity of the rearing environment affects adult cognition, which is essential for navigating complex environments. Dumontier et al. (2023) found that aviary-reared hens were faster than cage-

reared hens to first find bait in a hole board test, in which hens were required to use spatial cues to find bait in cups.

Brantsæter *et al.* (2016a) reared pullets either in cages or aviaries. These were transported to experimental pens containing perches and platforms at a variety of heights (70cm, 110cm, and 140cm above the floor level) at 16 weeks of age. Three-dimensional spatial use was observed in these birds at 19 weeks of age, and the aviary-reared birds used perches at all three height levels more than birds reared in cages. However, at 23 weeks of age, there was no longer a difference in perch usage between cage-reared and aviary-reared birds, suggesting that birds reared in a lower complexity environment do adapt to increased environmental complexity, albeit more slowly than those reared in a more complex environment. These birds were also tested in a combined human approach test and novel object test, and fear responses were lower for the aviary-reared birds than those reared in cages at both 19 and 23 weeks of age. Following the novel object tests, faecal samples were collected and analysed for corticosterone metabolites. There was no effect of rearing environment on the concentration of corticosterone metabolites at 19 or 23 weeks of age. Taken together, the findings of this study suggest that while increasing complexity in the rearing environment did not directly affect baseline HPA activity or fear-related behaviours during adulthood, it may be beneficial for welfare due to faster adaptation to a laying environment in which birds have more opportunity to use three-dimensional space.

Pullin *et al.* (2024) also found that the complexity of the rearing site affected adaptation to a multi-tier laying aviary. Pullets reared in either a floor system, a single-tier aviary, or a two-tier aviary were all moved into multi-tier aviaries at 17 weeks of age. Initially, higher use of the aviary tiers and more transitions between tiers during the evening were observed in birds reared in both aviary types than in floor-reared birds on the first day after transition to the multi-tier aviary, however these differences were not observed six days later. The time taken for floor-reared birds in Pullin *et al.* (2024) to reach the same spatial use as aviary-reared birds was shorter than the time taken for the cage-reared birds in Brantsæter *et al.* (2016a). It would be interesting to compare cages and floor systems directly. Even though the cage-reared pullets in Pullin *et al.* (2024) adapted to increased complexity within 1-2 weeks after transition to the laying site and showed similar aviary use to the aviary-reared birds across all tiers, the aviary-reared birds spent much more time in the highest tier of the aviary than cage-reared

birds, and this effect persisted through the last time-point at which spatial use was recorded at 27 weeks of age.

Even when moving to a less complex adult system, increased complexity during rearing appears to reduce fearfulness in adult hens. Brantsæter *et al.* (2016b) reared pullets in aviaries or enriched cages. When these were moved to enriched cages for laying, aviary-reared birds spent more time close to a novel object at 21 weeks of age than cage-reared birds. If increased complexity in early life is beneficial for welfare, this would support a "silver spoon" hypothesis (Grafen, 1988), in which individuals who experience lower adversity early in life may be more equipped to cope with adversity (such as changes in complexity) later in life. This is contrary to the general consensus that birds perform better in adult environments which are matched in complexity to the rearing environment (Janczak and Riber, 2015), which supports the "environmental matching hypothesis" (Montalcini, Petelle and Toscano, 2023), in which individuals are better adapted to environments that match early-life conditions, and even early-life adversity can play a positive role in promoting adaptation to the adult environment.

In order to evaluate the silver spoon hypothesis and the environmental matching hypothesis, Montalcini, Petelle and Toscano (2023) conducted a study comparing various welfare indicators, spatial behaviours, and production traits in adult laying hens which were either hatched on farm, or hatched at an external site then transported to farms at one day of age, which were all moved to laying aviaries at 17 weeks of age. They found that on farm hatched hens had higher severity of keel bone fractures, reduced vertical movement between aviary tiers in the first three months, and reduced time spent in littered areas than hens which were transported to the farm after hatching elsewhere. There was no difference in feather coverage or egg production between the treatments. They concluded that the results of this study align more closely with the environmental matching hypothesis. Pullin *et al.* (2024) suggests that rather than matching the environment exactly between the rearing and laying sites, it is the matching of behavioural opportunities which is important for adaptability.

Regardless of the level of environmental complexity, pullets are usually housed in systems which do not change throughout the rearing period, except by the introduction of perches early in the rearing period when they grow large enough to use them. Skånberg *et al.* (2023) suggested that adaptability to new environments later in life may be impaired by rearing in a fixed environment, and investigated whether adaptability can be improved by rearing in an environment in which the level of complexity regularly changes, or one in which birds are

presented with a choice of resources. They found that when birds were placed in a novel pen at 28-33 days of age, fear responses were reduced in birds that were reared in a changing environment. Also, birds that were able to choose the resources which they interacted with during rearing displayed less spatial clustering and more movement, suggesting increased motivation to explore novel environments, though the effects of choice and change were not additive. Together, the results of this study support the hypothesis that providing choice or change in the rearing environment can better prepare birds for their transition to a more complex laying environment.

The focus of the current study is how birds reared under the same conditions adapt to different adult environments, and whether this affects chronic stress or spatial cognition, as measured by hippocampal plasticity. Barnea and Nottebohm (1994) captured black-capped chickadees from the wild, and administered [^{3}H]-thymidine in order to label neurons that were newly incorporated into the hippocampus. Some of these chickadees were housed in a large, but relatively low-complexity aviary, while other birds were temporarily released back into the wild and allowed to range free. Some of the released chickadees were re-captured six weeks later, and brains from both the free-ranging and captive birds were collected to quantify [^{3}H]-thymidine-labelled neurons as a measure of neuronal recruitment. While new neurons were added to the hippocampus in both groups of birds, neuronal recruitment was much greater in the free-ranging birds which had the opportunity to explore a more complex environment.

In a similar study in zebra finches, Barnea, Mishal and Nottebohm (2006), juvenile birds were housed in small indoor cages from 45-60 days of age. Birds were then moved to larger outdoor aviaries at 4-5 months of age, where some birds were housed communally with conspecifics, while were housed singly. [^{3}H]-thymidine was administered, and brains were collected 40, 60, and 150 days after birds were moved to the larger aviaries. The density of labelled neurons was higher in the communally housed birds than singly housed birds in both the hippocampus and the nidopallium caudale (NC), suggesting that, in addition to a change in spatial complexity, changes in social complexity also affect neural recruitment. Taken together, the findings of these two studies show that neurons in the avian hippocampus are sensitive to both increases and decreases in environmental complexity from early life to adulthood. When moving to a more complex environment, there is a greater requirement for neuronal recruitment in order to form new spatial memories.

2.2.4. Functional gradient of the avian hippocampus

As previously mentioned, the mammalian dorsal DG in involved in processing of spatial information while the ventral DG is sensitive to emotional stimuli (Bannerman *et al.*, 2004). It has been hypothesised that there is a similar functional gradient along the rostro-caudal axis of the avian HF (Smulders, 2017). The rostral and caudal subregions of the avian HF have different proposed functions which are homologous to the mammalian dorsal and ventral DG respectively, and both subregions are relevant to the current study. The rostral HF has been proposed as the region which is most sensitive to spatial stimuli and has a role in spatial memory. In the free-ranging black-capped chickadees studied in Barnea and Nottebohm (1994), neuronal recruitment was greater in the rostral HF than caudal HF, while this was not observed in the captive birds. This enhanced neural recruitment in the rostral HF in response to greater environmental complexity supports the hypothesis that the rostral subregion is the area of the avian hippocampus that is most sensitive to changes in spatial stimuli.

Greater time spent on the range has been associated with increased expression of PCNA mRNA, which indicates higher cell proliferation, in the rostral HF but not in the caudal HF (Armstrong *et al.*, 2020b). The association between ranging and cell proliferation only in the rostral HF suggests that newly recruited neurons in the rostral HF may be required for navigating the increased complexity of the outdoor range, further supporting the hypothesised role of the rostral HF in spatial cognition.

The caudal HF is sensitive to emotional stimuli and plays a role in the stress response, and is the region in which effects on AHN have been observed in more severe stressors such as unpredictable chronic mild stress (Gualtieri *et al.*, 2019) and keel bone damage (Armstrong *et al.*, 2020a).

2.2.5. Aims and hypotheses

While previous studies have used DCX^+ cell density in the caudal HF to investigate the effects of more severe chronic stressors in hens (Gualtieri *et al.*, 2019; Armstrong *et al.*, 2020a), here, I aimed to investigate more subtle factors that could possibly contribute to chronic stress in laying hens by measuring DCX^+ cell density in both the caudal and rostral HF. Specifically, I were interested in the effects of the change in complexity from the rearing site to adult housing on hippocampal plasticity. This study compares hippocampal plasticity between laying hens housed in two different free-range housing systems: flat-deck (relatively low complexity, similar to the rearing site) and multi-tier (higher complexity than the rearing site).

Due to the proposed functional gradient along the rostro-caudal axis of the avian HF, I had separate hypotheses and predictions for each hippocampal subregion. Firstly, I hypothesised that hens housed in a more complex aviary experience higher spatial stimulation than those housed in a less complex system. If it is true that the multi-tier aviary provides greater opportunity to explore and therefore higher cognitive stimulation, I predict that multi-tier birds will show increased hippocampal plasticity in the rostral HF compared to flat-deck birds.

Secondly, I hypothesise that hens housed in a more complex aviary experience lower levels of chronic stress than those housed in a less complex system. If this is true, I predict that multi-tier birds will show increased hippocampal plasticity in the caudal HF compared to flat-deck birds.

In addition, I predicted that flat-deck housed birds would have a lower body mass at the end of life than multi-tier housed birds due to increased chronic stress caused by reduced environmental complexity. I also predicted that flat-deck birds would have a reduced spleen mass, due to reduced immune function caused by chronic stress.

2.3. Methods

2.3.1. Ethical statement

Ethical approval for this study was obtained from the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549).

2.3.2. Animals and housing

All birds used in this study were Shaver brown laying hens reared in the same shed, at a rearing site managed by The Lakes Free Range Egg company. At one day old, 22542 chicks were placed in the rearing site, which consisted of a large flat floor (total area 1707.4 m^2) with 13.2 birds per m^2 floor area, and a raised area for perching which was made available to pullets when they were large enough to use the perches. The lighting cycle was set to 10 hours light: 14 hours darkness per day, with an intensity of 12-15 lux when the lights were on. The pullets did not have access to an outdoor range during the rearing period. At 16 weeks of age, some of these birds were moved to a flat-deck housing system where they would be housed throughout adulthood, while the other birds were moved to a multi-tier aviary.

The flat-deck system had a similar design to the rearing site, and consisted of a large flat floor with a raised area containing some perches along one wall of the housing. In the multi-tier aviary, birds had access to a flat floor area in addition to a system of five tiers, which they

could move between freely. These two flocks were geographically close to one another and were both managed by The Lakes Free Range Egg Company. Both groups of adult hens were fed the same diet and had *ad libitum* access to water. The daily hours of lighting were gradually increased up to 15 hours light: 9 hours darkness per day. From 18-20 weeks of age onwards, pop holes were opened allowing access to an outdoor range during the daytime. The exact dates at which the pop holes were first opened are unknown for these flocks.

2.3.3. Selection of birds

Twelve birds were randomly selected from rearing site at 14 weeks of age. Selected birds were all close to average body mass and had no obvious differences in health or body condition to the rest of the flock. These birds were transported to Newcastle University where they were all housed in the same holding room, in which the lighting hours were set to match those of the rearing site. All birds were culled for tissue collection the next day.

Two weeks after the pullets were sampled, the remaining birds were moved to their adult housing systems. They were allowed to settle into the two adult housing systems for 8 weeks, before 12 birds were randomly selected from each adult housing system at 24 weeks of age. The flat-deck housing was separated into four large pens. Three average, healthy birds were randomly selected from each pen. Six of these were taken from the floor area, and six were taken from the raised perching area. The multi-tier aviary was also separated into four pens. In each pen, one bird was selected from the floor, one from a lower tier, and one from a higher tier.

In both adult systems, birds were palpated for keel bone damage (KBD), and were assessed using the simplified keel assessment protocol (SKAP), in which the presence or absence of fractures and deviations are reported separately and without a measurement of severity (Casey-Trott *et al.*, 2015). Four birds (two from the flat-deck, two from the upper level of the multi-tier) were rejected due to keel bone fractures or deviations that were detectable by palpation at 24 weeks of age. It is known that KBD affects hippocampal plasticity (Armstrong *et al.*, 2020a), therefore, rejecting birds with KBD of any type or severity level removes a source of unwanted variation from the study.

The birds from the two adult groups were identified using coloured leg bands and were transported to Newcastle University. The birds were all housed in the same holding room, in which the lighting was set to match the conditions of the sheds from which the birds were collected. One bird from each group lost their leg ring, and these two birds were excluded

from the study because there was no other way to identify which group they belonged to. Those two individuals were re-homed rather than being used for tissue collection. The remaining birds were culled for tissue collection over the following two days.

2.3.4. Tissue collection and immunohistochemistry

Pullets and adult birds were culled and dissected according to the same protocol. Birds were weighed then sedated with an intramuscular injection of ketamine (40mg/kg) and xylazine (8mg/kg). They were then euthanised by intravenous injection of an overdose of sodium pentobarbital. Immediately after death the brain was collected. One hemisphere of each brain was fixed in 4% paraformaldehyde for 48 hours at 4°C. The hippocampus was removed from the other hemisphere, and the rostral and caudal subregions were stored separately in RNAlater at -80°C for later analysis as part of a future study. A blood sample was collected post-mortem from each bird. The blood samples were centrifuged at 3000 rpm for 10 minutes to separate the plasma from the red blood cells. These samples were then frozen at -80°C for later analysis. The mass of the spleen from each bird was recorded, and a small piece of each spleen was stored in RNAlater at -80°C.

The fixed hemispheres were transferred to 30% sucrose in 0.1M phosphate buffered saline (PBS) solution for 48-72 hours (or until the brains sank to the bottom of the solution) at 4°C, then embedded in optimal cutting temperature compound (OCT) and stored at -80°C. 50µm coronal sections were cut on a cryostat. Serial sections at 400µm intervals were selected and were stained for DCX in six batches. In the first five batches, there were two brains from each treatment group. The sixth batch contained brains from the remaining two pullets, one flat-deck, and one multi-tier bird.

Free-floating sections were rinsed in 0.1M PBS, then endogenous peroxidase was inhibited by immersing the sections in 1% H_2O_2 solution for 30 minutes. The sections were then rinsed in 0.1M PBS, then incubated in blocking solution (1% bovine serum albumin, 2% goat serum, 0.3% Triton X-100 in 0.1M PBS) for 60 minutes. The sections were rinsed in with 0.1M PBS again, then incubated with a primary polyclonal antibody raised in rabbit against DCX (Abcam, Ab18723) at 4°C overnight (16 hours). The primary antibody was supplied at 0.9mg/ml and was used at a 1:1000 dilution in antibody buffer containing 2% goat serum and 0.3% Triton X-100 in 0.1M PBS.

The next day, sections were rinsed with 0.1M PBS then incubated with a secondary antibody (biotinylated anti-rabbit IgG raised in goat (Vector Labs, BA-1000) at 1:500 dilution in antibody buffer containing 2% goat serum and 0.1% Triton X-100 in 0.1M PBS) for 2 hours at room temperature. After being rinsed in 0.1M PBS, the sections were incubated with horseradish peroxidase streptavidin (Vector Labs, SA-5004) at 1:250 dilution in 0.3% Triton X-100 in 0.1M PBS for 60 minutes at room temperature. The sections were then rinsed again before being incubated in a solution of using SIGMAFAST 3,3'-Diaminobenzidine (DAB) tablets (Sigma-Aldrich, D4418) dissolved in ultra-pure water for 45 seconds.

Stained sections were rinsed in dH_2O, then stored in 0.1M PBS at 4°C before being mounted on subbed slides. When the slides were dry, they were immersed in 100% ethanol for 3 minutes. The slides were then transferred to a container of Histo-Clear (National Diagnostics, HS-200) for 5 minutes, then transferred to a second container of Histo-Clear for a further 5 minutes. Coverslips were applied to slides using Eukitt mounting medium (Sigma-Aldrich, 03989).

Seven brains (two pullets, three flat-deck, two multi-tier) were excluded from the study because the caudal HF was missing or damaged after staining. The remaining pullet (n=10), flat-deck (n=8) and multi-tier (n=9) brains were used for quantification of DCX^+ cell density.

2.3.5. Quantification of DCX^+ cells

From each brain, five sections containing rostral HF were selected at intervals of 800µm for quantification of DCX^+ cell density. One section containing caudal HF was also selected from each brain. In cases where more than one caudal section was stained, the largest and most caudal piece of HF was selected for quantification.

Cell counting was performed by a single observer. The slides were re-labelled with new ID numbers so that the observer was blind to the treatment groups. The slides were viewed through a Leica DMLB microscope with a ProScan II motorised stage (Prior Scientific, USA) and an attached camera (Optronics Microfire Digital Camera, USA). Cell counting was performed using the optical fractionator workflow in Stereo Investigator (MBF Bioscience, USA). In each section, a border was drawn around the perimeter of the HF at 2.5x magnification. The magnification was then increased to 100x for counting neurons. Within each counting frame (70µm x 70µm), stained bipolar and multipolar DCX^+ cells were counted. Bipolar cells were defined as those with fusiform cell bodies with two processes. Multipolar cells were defined as those with slightly larger cell bodies with three or more processes.

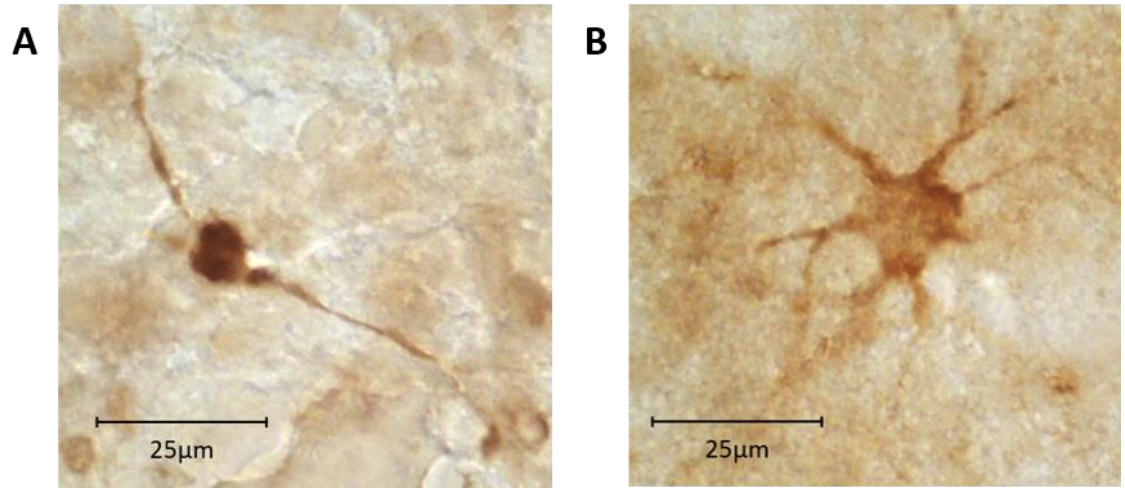

Figure 2.1 - Examples of DAB stained bipolar (A) and multipolar (B) DCX⁺ neurons in the hippocampus at 100x magnification.

The rostral and caudal HF were treated separately when calculating cell densities. The total volume sampled in each subregion of the HF was calculated by multiplying the number of sampling sites by the area of each counting frame (4900µm), multiplied by the cut thickness of the sections (50µm). The density of each cell type (cells per mm^3) was calculated by dividing the total number of each cell type by the total volume sampled in each subregion, then multiplying by 10^9. These cell densities were then Z-scored within staining batches to account for any differences between batches. Both cell types were Z-scored together and were analysed in the same model.

2.3.6. Statistical analysis

All statistical analysis was conducted using R Studio (R Core Team, 2023). The effects of the housing systems on DCX⁺ cell density was analysed using a three-way mixed ANOVA using the afex package (Singmann *et al.*, 2023), with housing group as a between subject factor, and subregion of HF and cell type included as within-subject factors.

The effect of the housing groups on body mass was analysed using a one-way ANOVA, and the effect of housing on spleen mass was analysed using an ANCOVA, with body mass included in the model as a covariate. Post hoc pairwise comparisons were conducted using the emmeans package (Lenth, 2024) in R to identify any differences between housing groups.

2.4. Results

2.4.1. Body and spleen mass

As shown in Figure 2.2, there was a significant difference in body mass between some housing groups ($F_{2,24} = 95.95$, $p < 0.001$). Post hoc pairwise comparisons show that the pullets were significantly lighter than the flat-deck housed birds ($p < 0.001$) and the multi-tier housed birds ($p < 0.001$), but there was no significant difference in body mass between the two adult groups ($p = 0.266$).

Figure 2.2 - Distributions of body mass (kg) at death for pullets (n=10), and adult laying hens housed in a flat-deck (n=8) or multi-tier (n=9) system. Black points and error bars represent mean ±1SE body mass.

After controlling for the effect of body mass, there was no significant difference in spleen mass (Figure 2.3) between the housing groups ($F_{2,23}$ = 1.45, p = 0.255).

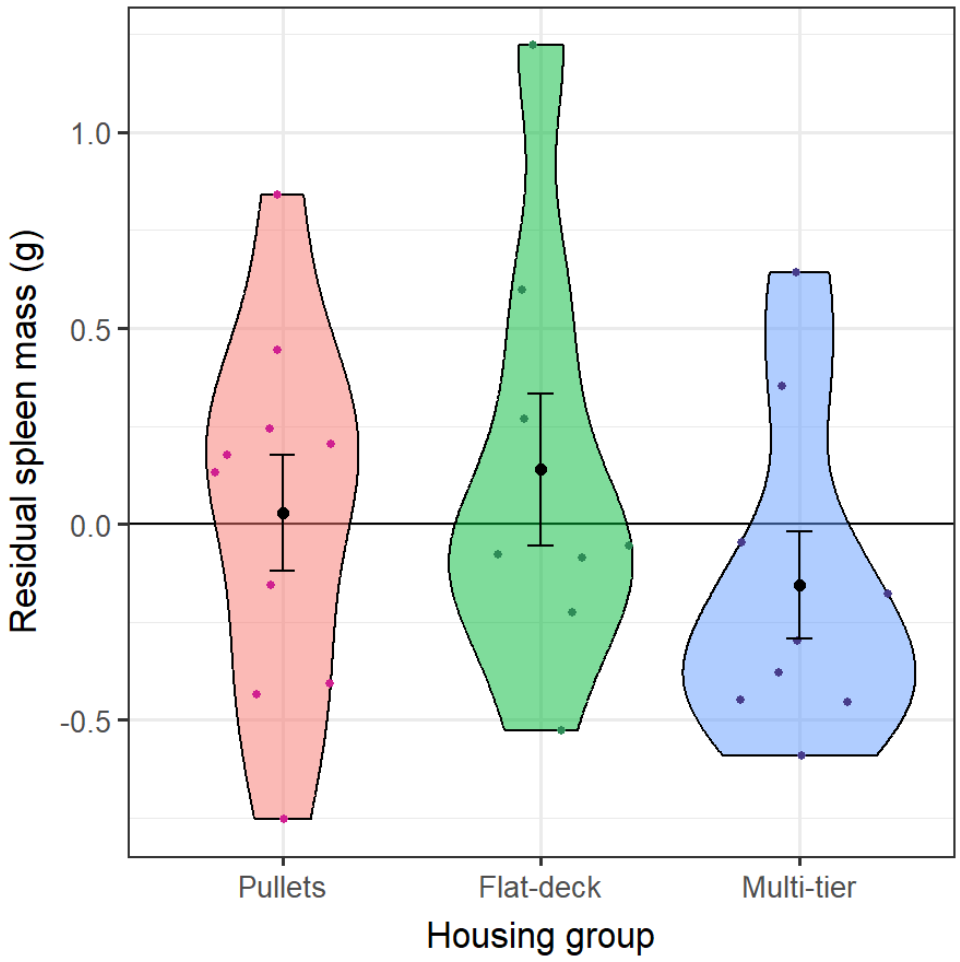

Figure 2.3 - Distributions of residual spleen mass (after correction for body mass at death) for pullets (n=10), and adult laying hens housed in a flat-deck (n=8) or multi-tier (n=9) system. Black points and error bars represent mean ±1SE residual spleen mass.

2.4.2. Effects of housing systems on DCX⁺ cell density

There was no significant main effect ($F_{2,24}$ = 0.18, p = 0.835) of housing group on DCX⁺ cell density (Figure 2.4), and no significant interaction between housing group and cell type ($F_{2,72}$ = 0.41, p = 0.666) or subregion of HF ($F_{2,72}$ = 1.70, p = 0.190). Overall, there was a significantly higher density of bipolar DCX⁺ cells than multipolar DCX⁺ cells ($F_{1,72}$ = 252.28, p <0.001). There was also a main effect of subregion of HF, with significantly higher DCX⁺ cell density in the caudal HF than the rostral HF ($F_{1,72}$ = 1.032, p = 0.001).

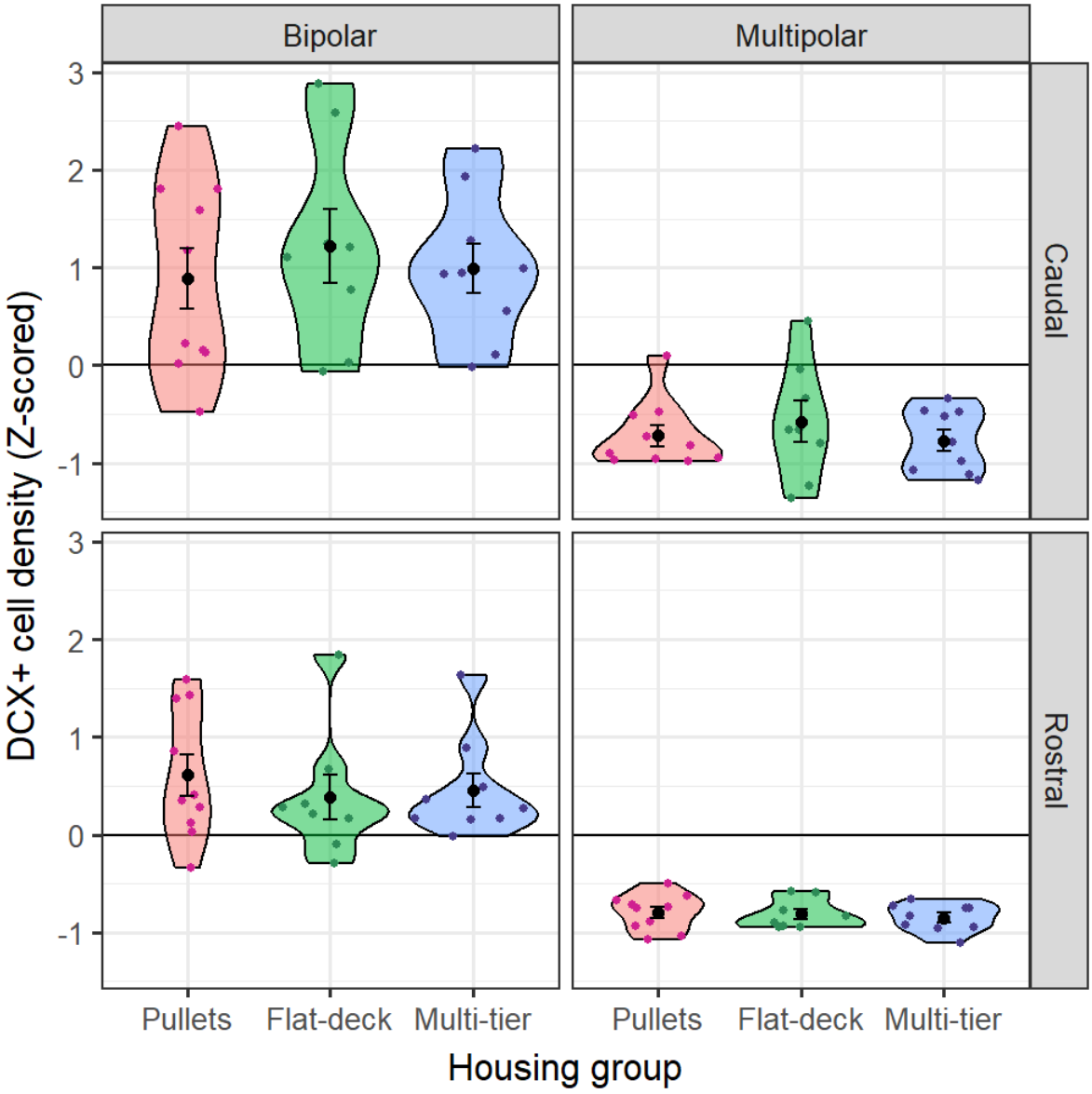

Figure 2.4 – Distributions of the densities of bipolar and multipolar DCX⁺ cells (z-scored within staining batches) in the rostral and caudal HF of pullets (n=10) and adult hens housed in a flat-deck (n=8) or multi-tier (n=9) system. Black points and error bars represent mean ±1SE DCX⁺ cell density.

There was a significant interaction between cell type and subregion of HF on cell density ($F_{1,72}$ = 4.39, p = 0.040). Pairwise comparisons show there was a significantly higher density of bipolar DCX⁺ cells in the caudal HF than the rostral HF ($p < 0.001$), but no significant difference in multipolar DCX⁺ cell density between the caudal and rostral HF (p = 0.388). The three-way interaction between the effects of housing group, cell type, and subregion of HF on DCX⁺ cell density was not significant ($F_{2,72}$ = 0.12, p = 0.891).

2.5. Discussion

There were no significant differences in DCX^+ cell density between the housing groups in either the rostral or caudal HF. Any differences between the multi-tier and flat-deck systems were not great enough to have a detectable effect on hippocampal plasticity.

The higher density of bipolar DCX^+ neurons across the whole HF compared to multipolar DCX^+ neurons was expected. Bipolar cells are the least developed neurons, while multipolar neurons are more mature and may have started to differentiate (Balthazart and Ball, 2014). The increased density of bipolar DCX^+ neurons in the caudal HF compared to the rostral HF was similar to the findings of Armstrong *et al.* (2022) in adult caged and free-range hens. However, they also found a higher density of multipolar DCX^+ neurons in the caudal HF compared to the rostral HF, which I did not find in the current study. This may be due to the different housing environments used, the age of the birds (65-66 weeks of age at sampling in Armstrong *et al.* (2022), compared to 12-24 weeks of age in the current study), or the different strains of birds (H&N and Hy-Line) that were used in the previous study.

2.5.1. Body and spleen mass

The significant main effect of body mass between the housing groups was expected, because the pullets were 14 weeks of age at the time of sampling, and were therefore smaller and lighter than the birds that were sampled from the adult systems at 24 weeks of age. The lack of difference in body mass between the two adult housing groups suggest that any chronic stress experienced due to housing was not severe enough to affect growth in either group, and the different changes in complexity from rearing site to adult housing did not affect growth.

There was no difference in residual spleen mass between any of the groups, which also supports the finding that there was no difference in chronic stress between the groups. Spleen mass decreases in chickens when exposed to chronic stress, as demonstrated by Puvadolpirod and Thaxton (2000) using continuous ACTH administration. The lack of difference in spleen mass also suggests no difference in immune function between birds housed in these systems.

2.5.2. Rostral HF

While there appears to be a great difference in spatial complexity between the flat-deck and multi-tier housing, this was not reflected in plasticity in the rostral HF. the lack of difference in plasticity in the rostral HF between pullets and each of two adult housing groups suggests that there was no difference in the change in complexity between the rearing site and each

adult housing system. Therefore, the findings of the current study do not support my hypothesis that the birds in the more complex aviary would experience higher spatial stimulation. The spatial challenges presented to birds by the multi-tier aviary may not have been as extreme, or at least not as different to the flat-deck system, as was expected.

Both groups of adult birds had access to an outdoor range for at least 4 weeks before brains were sampled, which may be a reason why their spatial experience was more similar than expected. Plasticity in the rostral HF at 24 weeks of age may have been influenced by adaptation to the novel spatial experience of being allowed to use the range, which was common between both groups of adults, rather than being influenced by the earlier change in housing complexity that I intended to investigate. It is possible that access to the range may also have affected results in the caudal HF because of possible welfare benefits of increased opportunity to explore. In a future study, it may be interesting to sample brains from the same adult systems before they given access to the range, though it is unclear whether differences in hippocampal plasticity would develop within the 2-4 weeks between transfer to laying aviaries and pop-holes being opened. It may also be interesting to investigate whether range use differs between birds which were reared together then moved to laying systems with differing complexity inside the barn.

Pullets have been found to adapt more quickly when introduced to more complex housing systems at an earlier age (17 weeks vs 25 weeks) (MacLachlan *et al.*, 2020), therefore it is possible the birds, which were transferred at 16 weeks of age in the current study, may have adapted to their respective housing systems very quickly, so there was no detectable change in hippocampal plasticity caused by adaptation to their new environments by the time the brains were sampled. The birds sampled as pullets also had 12 weeks since their last novel spatial experience as they were introduced to the rearing site at one day of age, so it is likely that they also had time to adapt to the complexity of their environment in the weeks before brains were sampled. This may explain the lack of difference between the pullets and either adult group.

2.5.3. Caudal HF

The lack of difference in hippocampal plasticity in the caudal HF between the flat-deck and multi-tier aviaries does not support my hypothesis that hens housed in a more complex aviary experience lower levels of chronic stress than those housed in a less complex system, and

suggests that any welfare differences between these two housing systems were not great enough to have a detectable effect on plasticity in the caudal HF.

The findings of this study were similar to those of Armstrong *et al.* (2022), in which there was also no effect of environmental complexity on the density of DCX^+ neurons across the hippocampus. In that study, there was an even greater difference in complexity in the laying environment than in the current study, as the multi-tier birds were compared to a group of caged hens rather than birds from a less complex cage-free system. Therefore, the lack of effect of environmental complexity on hippocampal plasticity in the current study is not surprising. Armstrong *et al.* (2022) also investigated differences between birds with good and poor body condition, and regardless of the level of environmental complexity, there were significantly lower multipolar DCX^+ neurons in the HF of poor condition birds than good condition birds, and a trend towards fewer bipolar DCX^+ neurons in the HF of poor condition birds compared to good condition birds.

Following the current study, more brains were taken from the same laying flocks when the birds were 60-61 weeks of age. In this sample, 12 good body condition and 12 poor body condition birds were selected from each flock. It could be interesting to investigate the effects of body condition and interactions between body condition and housing on the brains of birds taken from these more similar environments. It would also be interesting to use the brains from the later sample to investigate whether differences in hippocampal plasticity developed after 44-45 weeks after moving to the different laying systems. While 8 weeks of chronic stress has been found to affect hippocampal plasticity previously (Gualtieri *et al.*, 2019), and this was the same amount of time that the adult birds spent in the laying systems, more subtle putative stressors such as low environmental complexity may require a longer period of exposure in order to have a detectable effect on hippocampal plasticity. Although, Armstrong *et al.* (2022) did not find an effect of housing system in birds at 65-66 weeks of age.

In conflict with these studies in chickens, there has been evidence that DCX immunoreactivity is increased in mice in response to increased environmental complexity (two tiered cage with tunnels and running wheel vs single tiered barren cage) for 4 weeks (Terada *et al.*, 2008) though this effect was not specific to the ventral DG. When some of those mice were exposed to chronic pain by nerve ligation, the effects of environmental complexity on DCX were suppressed. It is possible that in the current study there may have been an unknown source of stress in the multi-tier aviary which eliminated any positive effect of complexity on

hippocampal plasticity in a similar manner to chronic pain in mice, though further study would be required to determine if this was the case, and steps were taken to remove known sources of stress such as KBD.

Alternatively, both adult systems may have been high-welfare. Though I expected better welfare in the more complex environment based on studies in rodents, the environmental matching hypothesis, supported by studies including Montalcini, Petelle and Toscano (2023), suggests that a matched environment is also good for welfare. The flat-deck, which I predicted to be the lower welfare system, was more closely matched with the design of the rearing site than the multi-tier aviary. If a matched environment and an enriched environment both have similar effects in reduce chronic stress, this may be a reason for the lack of difference in hippocampal plasticity in the caudal HF between the two systems.

Rather than informing us about welfare differences between two housing systems, the findings of this study may be more informative about the efficacy of hippocampal plasticity as a method to quantify the effects of chronic stressors which are subtle compared to more severe chronic stressors in which effects in the caudal HF have been observed previously (e.g. Robertson *et al.*, 2017; Gualtieri *et al.*, 2019; Armstrong *et al.*, 2020a). Subtle differences such as housing design may require a more sensitive method than hippocampal plasticity in order to accurately quantify chronic stress.

2.5.4. Study limitations

With the uncertainty concerning the effects subtle differences such as housing complexity on hippocampal plasticity, it would have been useful to measure other welfare indicators between the housing systems that were not detected by looking at the brain alone. This may have included measuring blood plasma corticosterone, body condition or feather scoring, or measuring fear or anxiety-related behaviours in tests such as novel object tests.

It would have been ideal to sample birds from more than one flock from each housing type, though this would have been difficult logistically. Within the same category of adult housing, there may be variation between flocks, and even subtle variations between the design of individual sheds within each system type which may affect welfare. For example, Heerkens (2016) found that differences in floor type in multi-tier systems affected plumage score, which is an established welfare indicator.

One major disadvantage of using hippocampal plasticity to quantify stress is that it is impractical to process a large number of brains. Damage to the HF during processing of sections reduced sample size, which was already quite small with only 12 birds per group. Also, only one section of caudal HF was counted for each bird. While the largest section of the caudal HF was used wherever possible, a single section may not be representative of the whole caudal HF.

2.5.5. Conclusions

In conclusion, the results of this study suggest any differences in welfare between birds housed in flat-deck compared to multi-tier systems would be minimal. The difference in complexity between the flat-deck and multi-tier aviaries was not great enough to have a detectable effect on hippocampal plasticity in either the rostral or caudal HF. Therefore, the findings of the current study do not support my hypotheses about the effects of environmental complexity on either subregion of the HF. If there were any differences between the housing systems, they were not severe enough to be reflected in the brain and are therefore not as severe as other stressors that have been studied previously.

This study did not find any evidence that replacing flat-deck housing units with multi-tier aviaries would either decrease or increase the severity of chronic stress experienced by hens. However, each of these housing systems may have associated welfare considerations that were not investigated in this study, and there may still be practical benefits for choosing to use multi-tier aviaries.

Chapter 3. Effects of footpad dermatitis on hippocampal plasticity and the caecal microbiome of laying hens

3.1. Abstract

Footpad dermatitis (FPD) is a common health condition in commercially housed hens. Birds with severe FPD have necrotic lesions on the feet, which are caused by prolonged contact with wet bedding housing environment. FPD is believed to have a negative impact on the welfare of laying hens, and may therefore affect the efficiency and sustainability of egg production. Hens managed under semi-commercial conditions were scored on three occasions for FPD severity. Birds with the most severe FPD (n=12) were selected to be compared with control birds (n=9). In each of these groups, some birds were reared with a dark brooder while others were not. Selected individuals were culled at 70 weeks of age, and their brains were collected and dissected. One hemisphere of each brain was fixed and then cut to 50µm coronal sections, which were immunohistochemically stained against doublecortin (DCX), a marker of neural plasticity, in order to quantify hippocampal plasticity as a measure of chronic stress. I predicted that FPD would cause a decrease in DCX^+ cells in the hippocampal formation (HF) of conventionally reared birds, and that the effects of FPD on DCX^+ cell density would be smaller or non-existent in birds reared with a dark brooder. In conventionally reared birds, FPD tended to decrease DCX^+ cell density across the HF, but in dark brooder reared birds, FPD tended to increase DCX^+ cell density across the HF. These results suggest that FPD is chronically stressful, and rearing birds with a dark brooder could increase resilience to stress from FPD.

The composition of the caecal microbiome was also compared between groups using 16S rRNA sequencing. There were no differences significant in alpha or beta diversity distributions between the groups, though there was an increased abundance of some genera of the family Veillonellaceae in FPD birds compared to controls.

3.2. Introduction

Hens commonly experience injuries and illnesses that are related to their housing conditions which may cause chronic stress. Here, I aim to investigate the effects of a common health problem, footpad dermatitis (FPD) on both the brain (hippocampal plasticity) and the caecal microbiome.

3.2.1. Footpad dermatitis

FPD is common in commercially housed hens, in which birds have necrotic lesions and swelling on the feet. This is sometimes referred to as “pododermatitis”, or “bumblefoot” in more severe cases where swelling of the footpad is dorsally visible (Wang, Ekstrand and Svedberg, 1998). FPD has mainly been studied in broilers, but is also a significant problem in laying hens (Alberghina *et al.*, 2020). Estimates of the prevalence of FPD in laying flocks vary greatly, though in part this could be due to differing terminology used to describe footpad pathologies. For example, Heerkens (2016) found a mean prevalence of 27.6% hens with FPD in free-range aviaries, though this was not the only category of foot disorder studied, and 42% hens were found to also have hyperkeratosis of the footpads, and “bumblefoot” was scored as a separate condition reported in only 1.2% hens. Weitzenbürger *et al.* (2006) reported footpad pathologies in 86.1% laying hens, which ranged from mild hyperkeratosis to deep lesions and severe swelling.

Lesions often become infected by pathogenic bacteria that can cause systemic infections (Chen, Tellez and Escobar, 2016) and are therefore implicated in food safety. Heidemann Olsen *et al.* (2018) collected bacterial samples from foot lesions from hens with severe FPD, and used 16S rRNA sequencing to identify the most abundant bacteria. *Staphylococcus aureus* was found to be the most abundant bacterial species in FPD lesions, followed by *Enterococcus faecalis and Escherichia coli.* Heidemann Olsen *et al.* (2018) suggest that many birds may be infected from a common source, because *S. aureus* is ubiquitous in poultry housing.

Pathogens causing inflammation can enter the footpads through FPD lesions caused by a range of factors. Prolonged contact with wet bedding is believed to be the most significant factor in the development of FPD lesions (Wang, Ekstrand and Svedberg, 1998). FPD is also associated with a number of other factors in commercial poultry housing including high stocking density and poor ventilation (Shepherd and Fairchild, 2010). Early life experience has been found to affect FPD, with higher prevalence and severity of FPD at 21 and 35 days of age in broilers hatched in a hatchery compared to those hatched on farm (Giersberg *et al.*, 2021), though other animal based welfare indicators (plumage cleanliness, skin lesions, gait score) were not affected by hatching system and it is unclear why FPD was affected.

FPD is believed to be painful, evidenced by the effect of FPD on behavioural indicators of pain such as gait instability (Hester, 1994), and is therefore believed to have a negative impact on the welfare of hens.

3.2.2. Dark brooders

During rearing, chicks do not have access to their mother which limits their ability to express some natural behaviours. A dark brooder is a horizontal heating element surrounded by a dark curtain, which provides a warm and dark place for birds to shelter. These are used on some rearing farms to simulate the presence of the mother during the first few weeks of life, and are believed to have welfare benefits which persist into adulthood, including a reduction in damaging behaviours and consequently better body condition (Jensen, Palme and Forkman, 2006). In a review of existing studies on the welfare benefits of dark brooders conducted by Sirovnik and Riber (2022), it was reported that behavioural studies conducted on birds at a range of ages (4-26 weeks) have found reduced fearfulness in those reared with a dark brooder. The age at which dark brooders were removed from the chicks varied greatly in these studies, from 5-16 weeks of age, however it is clear that the reduced fear responses persisted long after dark brooders were removed.

Feather pecking, a behaviour observed in poultry flocks that may be influenced by stress, has been compared between five flocks in which chicks were reared conventionally, and five flocks in which chicks were reared with a dark brooder (Gilani, Knowles and Nicol, 2012). Feather pecking was quantified in all flocks at 1, 8 and 16 weeks of age, and in three flocks from each rearing condition at 25 and 35 weeks of age. Across all ages tested, the dark brooder flocks had less severe feather pecking. Birds reared with a dark brooder showed greater social reinstatement during novel environment tests (de Jong *et al.*, 2022), meaning the birds showed higher motivation to be reunited with conspecifics during the tests. This may be due to reduced aggressive or injurious behaviours (such as feather pecking) between dark-brooder reared birds. de Jong *et al.* (2022) also found that birds reared with a dark brooder had better FPD scores than those reared conventionally, though the severity of chronic stress experienced by dark brooder reared birds which do have severe FPD remains unknown.

3.2.3. Gut-brain axis

In recent years, many studies have investigated the bi-directional “gut-brain axis”, in which communication between the gut and the brain is implicated in the physiology of healthy individuals and those suffering from pathological conditions (Hajjo and Geva-Zatorsky, 2019). The gut microbiota may cause changes in behaviour, and the conditions such as chronic stress may change the composition of the gut microbiome. Two-way communication using the vagus nerve, immune signalling, and a “leaky gut”, in which bacteria and their metabolites cross the

epithelial layer of the gastrointestinal tract, have been proposed as the main pathways for this regulation of stress by the gut microbiota (Foster, Rinaman and Cryan, 2017), mostly in studies using germ free mice. It has also been shown that the gut microbiome regulates neurogenesis (Ogbonnaya *et al.*, 2015), especially in the dorsal hippocampus.

The composition of the gut microbiome has also been proposed as a possible contributing factor to chronic stress in chickens. For analysis of the gut microbiome in chickens, samples are most commonly taken from the caecum, because this region of the gastrointestinal tract has the highest density of bacterial cells (Andreani, Donaldson and Goddard, 2020), therefore high biodiversity. The caecum also has a greater role in digestion in birds than in most mammals, meaning the caecal microbiota have a more significant influence on the performance and overall health of the animal (Stanley *et al.*, 2015). Therefore, sequencing the caecal contents may provide information about production in addition to welfare.

The regulation of stress by microbiota has been investigated in chickens in studies using behaviours which are believed to be associated with stress. For example, differences in the caecal microbiome have been found between laying hens divergently selected for high or low levels of feather pecking (van der Eijk *et al.*, 2019). High feather pecking (HFP) birds had higher biodiversity, higher relative abundance of genera in Clostridiales, and lower relative abundance of *Lactobacillus* than low feather pecking (LFP) birds. It is unclear whether these differences in the microbiome were caused by increased feather pecking, or if changes in microbiota due to an unknown cause contributed to increased feather pecking. Borda-Molina *et al.* (2021) also found differences in microbiome composition between HFP and LFP hens, but did not find that taxa which were differentially abundant between the strains were causative of feather pecking behaviour.

The causative effects of the gut microbiota on host behaviour and physiology can be investigated by transplantation of microbiota. Using samples which were collected from the lumen of the gut in HFP and LFP hens during their previous study (van der Eijk *et al.*, 2019), chicks from the same divergently-selected lines received transplants of microbiota from HFP or LFP birds, or a saline control, which was administered orally every day for the first two weeks post-hatch (van der Eijk *et al.*, 2020). Compared to birds which received microbiota from the other line or a control treatment, those which received microbiota from their own line showed more active responses in behavioural tests such as decreased latency to approach a novel object or vocalise during an open field test, though there was no significant effect of

transplantation on feather pecking directly, and no effect on plasma corticosterone after manual restraint. Overall, that study suggests that early-life colonisation of the gut has an effect on stress-related behaviours, and that the effect of the microbiome on stress-related behaviours is dependent on the genetic predisposition of the host.

The intestinal tract of a chick is colonised by environmental bacteria immediately after hatching (Richards-Rios *et al.*, 2020b), which may include pathogens and species which have functions in regulating the stress response. Therefore, rearing in an environment which promotes healthy development of the gut microbiome may play an important role in stress resilience during adulthood. Laying hens reared in caged and cage-free systems have been found to have different populations of caecal microbiota at 120 days of age (Shi *et al.*, 2019). Hens reared in cage-free systems had a relatively higher abundance of Spirochaetaceae (of unclassified genus), while hens reared in cages had relatively higher abundance of the genera *Faecalibacterium*, *Ruminococcaceae*, and *Helicobacter*.

Even within cage-free systems, the design of the early-life environment may affect the caecal microbiome. Complexity may be added to cage-free rearing environments using perches at varying heights to allow greater use of three-dimensional space, or novelty may be provided by regularly changing enrichment items. Bari *et al.* (2022) compared the caecal microbiome between hens reared in barren pens with those reared with structural enrichment (access to perches) or novel enrichment (items such as balls, bottles and pet toys which were changed every week). At 15 weeks of age, they found differences in beta diversity (a measure of the differences in the composition of the microbiome between samples) between the three rearing treatments, but no differences in alpha diversity (a measure of the diversity of taxa within an individual sample). At 16 weeks of age, all remaining pullets were moved to identical adult housing pens in an experimental facility with access to an outdoor range. At 65 weeks of age, birds were sampled for caecal microbiome sequencing, and it was found that the differences in microbiota observed between the pullets from different rearing conditions persisted into adulthood, even though the environmental differences were not present during the production phase.

While the caecal microbiome seems to be most sensitive to changes that occur during early life, differences have also been detected in adult hens housed in different conditions. Adhikari *et al.* (2020) sampled caecal microbiota late in the production phase (between 53 and 72 weeks of age) from two strains of laying hens (Hy-Line W36 and Hy-Line Brown) housed in

conventional or enriched cages. Both strain and cage type had significant effects on the composition of the caecal microbiome and the functional pathways in which differentially abundant bacteria were involved.

The caecal microbiome of adult hens has also been compared between those housed in enriched cages and free-range multi-tier aviaries. There was a significant difference in beta diversity between the two housing groups, and body condition significantly affected beta diversity in multi-tier birds, but not in caged birds (Armstrong *et al.*, 2022). Across adult hens housed in both enriched cages and free-range multi-tier aviaries, Armstrong *et al.* (2022) also found higher abundance of methanogens, specifically the archaea *Methanomethylophilaceae*, in the intestinal tract of birds with poor body condition compared to those with good body condition. The high abundance of methanogens may have been contributing to poor body condition, thereby causing chronic stress which was detectable using hippocampal plasticity.

3.2.4. Aims and hypotheses

FPD is both painful and a source of pathogenic infections, and is therefore an example of a housing-related health condition which we may expect to affect both hippocampal plasticity (see Chapters 1 and 2) and the composition of the caecal microbiome. The main aim of the current study was to investigate the severity of FPD as a chronic stressor in laying hens by quantifying hippocampal plasticity in hens with varying degrees of FPD. I hypothesised that birds with severe FPD experience chronic stress. It was predicted that hens with severe FPD would have reduced density of DCX^+ cells in the caudal HF compared to control birds in which symptoms of FPD were absent or minimal. It was also predicted that as cumulative FPD severity (scored across three time-points) increased, the density of DCX^+ cells in the caudal HF would decrease.

I also aimed to investigate the effects of FPD on the caecal microbiome of laying hens using 16S rRNA sequencing. I hypothesised that chronic stress caused by FPD changes the composition of the caecal microbiome, though I had no strong predictions about differences in the abundance of specific taxa between FPD and control birds.

I also measured the body mass and brain mass of the birds. I predicted that if FPD causes chronic stress, FPD birds would have lower body mass than controls, and may have lower brain mass than controls.

The effects of dark brooding and KBD and their interactions with FPD were also explored, though these were not originally intended as aims of the current study, therefore I have no specific hypotheses about the effects or rearing and KBD. Nevertheless, I predict that KBD, as a known chronic stressor in birds, will cause a decreased density of DCX^+ neurons at the caudal pole of the HF, and will affect the composition of the caecal microbiome. Based on previous studies suggesting that dark brooders may confer resilience to chronic stress, I predict that the effects of FPD on DCX^+ cell density in dark brooder reared birds will be smaller than those observed in conventionally reared birds.

3.3. Methods

3.3.1. Ethical Statement

Ethical approval for this study was obtained from the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549). This study also complied with ethical standards at Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Belgium, where animals were housed and tissues were collected.

3.3.2. Animals and housing

NOVOgen Brown Light laying hens (n=21) were selected from a study conducted at ILVO, and according to local ethical approval on the use of animals in research. All birds were reared in the same poultry house in two pens, one of which contained dark brooders which were present for the first 8 weeks of life. Adult hens were housed in one of four sheds, each containing 51 birds. The birds in two of these sheds had been reared with a dark brooder, while the other birds in the other two sheds had been reared conventionally (without a dark brooder). Each group had access to an outdoor range measuring 90m x 10m, which contained grass and trees.

3.3.3. FPD severity scoring

Hens were scored for FPD severity and selected by Dr Elske De Haas, who was trained in FPD assessment. The hens were scored for FPD severity on a scale of 0-2 (Figure 3.1) on three occasions (May, July, and September 2019) according to the Welfare Quality Assessment Protocol for Laying Hens (van Niekerk, Gunnink and van Reenen, 2012). Only individuals that scored 0 for FPD on at least two sampling occasions (including September) were selected as control birds (n=9). Birds which had a score of 2 on all three occasions, or a score of 1 in May and 2 in July and September were selected as those with the most severe FPD (n=12).

Score	Description	Example
0	Feet intact, no or little proliferation of epithelium	
1	Necrosis or proliferation of epithelium or chronic bumblefoot with no or moderate swelling	
2	Swollen (dorsally visible)	

Figure 3.1 - Criteria for severity scores based on the Welfare Quality Assessment Protocol for Laying Hens (van Niekerk, Gunnink and van Reenen, 2012)

All control birds had a September score of 0, and all FPD birds had September score of 2. In each group, 4 birds were not reared with a dark brooder (Table 3.1). For each individual, a cumulative score (0-6) was calculated by adding together the scores recorded in May, July and September.

3.3.4. Tissue collection and immunohistochemistry

At 70 weeks of age, the selected birds were weighed then sedated with an intramuscular injection of ketamine (40mg/kg) and xylazine (8mg/kg) before being euthanised by intravenous injection of sodium pentobarbital. Dissections were conducted over two days (9 birds on day 1, 12 birds on day 2). During dissections, keel bones were removed and any instances of KBD were recorded. Out of 21 birds, 10 individuals were reported to have some degree of KBD (Table 3.1), mostly at the caudal tip of the keel bone.

Table 3.1 - Number of sampled birds with and without KBD in each treatment group

	With KBD	**Without KBD**	*Row totals*
FPD Dark Brooder	3	5	8
FPD Conventional	2	2	4
Control Dark Brooder	3	2	5
Control Conventional	2	2	4
Column totals	10	11	21

One caecum was collected from each bird and was immediately placed in a collection tube on dry ice, before subsequent storage at -80°C. The total mass of each brain was recorded, then one hemisphere of each brain was fixed in 4% paraformaldehyde for 48 hours at 4°C. The hemispheres were then transferred to 30% sucrose in 0.1M phosphate buffered saline (PBS) solution for 48-72 hours, then embedded in optimal cutting temperature compound (OCT) and stored at -80°C.

The fixed hemispheres were cut to 50µm coronal sections on a cryostat. Serial sections at 200µm intervals were selected for immunohistochemistry. The sections were stained according to the same protocol which is described in full in chapter 2, but briefly, free-floating sections were immersed in 1% H_2O_2 solution to inhibit endogenous peroxidase. The sections were then incubated in a primary polyclonal antibody raised in rabbit against DCX (Abcam, Ab18723) at 1:1000 dilution, then in a secondary biotinylated anti-rabbit IgG raised in goat (Vector Labs, BA-1000) at 1:500 dilution. The sections were incubated in a solution containing the conjugate enzyme horseradish peroxidase streptavidin (Vector Labs, SA-5004) at 1:250 dilution, then stained in a solution made with SIGMAFAST 3,3'-Diaminobenzidine (DAB) tablets (Sigma-Aldrich, D4418) in ultra-pure water before being mounted on slides. The sections were stained in 5 batches of 3-7 brains with FPD and control birds represented in each batch, though rearing and KBD were not considered when assigning brains to staining batches. All personnel involved in staining were blind to the treatment groups to which each brain belonged.

3.3.5. Quantification of DCX$^+$ cells

From each brain, five sections containing rostral HF were selected at intervals of 800µm for quantification of AHN. The section containing the largest piece of the caudal HF was also selected from each brain. The slides were viewed through a Leica DM-LB microscope with a ProScan II motorised stage (Prior Scientific, USA) and an attached video camera (Optronics

Microfire Digital Camera, USA). Cell counting was performed by a single observer who was blind to the treatment groups, using the optical fractionator workflow in Stereo Investigator (MBF Bioscience, USA). The full details of the cell counting protocol are the same as those described in full in chapter 2.

The densities of bipolar and multipolar DCX^+ neurons were calculated separately in the rostral and caudal HF. Raw cell densities (cells per mm^3) were Z-scored within each bird (both cell types and hippocampal subregions together) to account for any differences between staining batches.

One brain (from an FPD bird, reared conventionally) was damaged during dissection, and was therefore removed from the analysis. Another two brains (both from the FPD group, one conventional, one dark brooder) were missing the caudal HF after staining. Cell densities were still quantified for these individuals in the rostral HF only, and they were included in the analysis. still Final sample sizes for each treatment group for analysis of DCX^+ cell density can be found in Table 3.2. The damage occurred after brains were weighed, so all 21 birds were included in the analysis of brain mass.

Table 3.2 - Number of birds in each treatment group for analysis of DCX^+ cell density after excluding individual with damaged brain. Numbers in brackets () show the number of birds for which only the rostral HF was present.

	With KBD	**Without KBD**	*Row totals*
FPD Dark Brooder	3 (2)	5	8 (7)
FPD Conventional	2 (1)	1	3 (2)
Control Dark Brooder	3	2	5
Control Conventional	2	2	4
Column totals	10 (8)	10	20 (18)

3.3.6. Caecal microbiome

Analysis of the caecal microbiome was conducted at the University of Liverpool under the supervision of Professor Paul Wigley and Dr Sian Pottenger. Caecae were thawed at room temperature, then a small incision was made at the distal end of each caecum. A sterile inoculating loop was used to transfer 200mg caecal contents from each sample into ZR BashingBead lysis tubes (Cambridge Bioscience, UK), which were shaken using a Qiagen TissueLyser for 10 minutes in order to lyse the bacterial cell walls. In addition to the caecal samples, two microbial community standards (Cambridge Bioscience) and a negative control

were also included. DNA was extracted from samples using a ZymoBIOMICS DNA Miniprep Kit (Cambridge Bioscience, D4300) according to the manufacturer's instructions. A Qubit fluorometer (Invitrogen, UK) was used to quantify the concentration of extracted DNA in the samples. Concentrations of DNA ranging between 2.2ng/µl – 54.0ng/µl were obtained. All samples were diluted to a standardised concentration of 10ng DNA in 10µl nuclease-free water. The 16S rRNA gene was then amplified using PCR.

After amplification, DNA concentrations were quantified again, then diluted to produce samples with a standardised concentration of 100ng DNA in 0.4µl nuclease-free water. These samples were carried forward for 16S rRNA sequencing, using a FLO-MIN106 flow cell (Oxford Nanopore Technologies, UK) and a 16S barcoding kit (Oxford Nanopore Technologies, UK, SQK-16S024) according to the manufacturer's instructions. Reads obtained from 16S rRNA sequencing were processed by Dr Rebee Penrice-Randal for taxonomic classification using Kraken (Wood, Lu and Langmead, 2019). The output from Kraken was formatted into a biological observation matrix (BIOM) file containing data about the operational taxonomic units (OTUs) that were identified in each sample at genus level. The BIOM file was merged with sample metadata, and this was used for downstream analysis of features of the caecal microbiome using the 'phyloseq' package in R (McMurdie and Holmes, 2013), including alpha diversity, beta diversity, and differential taxa abundance.

Table 3.3 – Number of birds in each treatment group for analysis of caecal microbiome after excluding outliers

	With KBD	**Without KBD**	*Row totals*
FPD Dark Brooder	3	4	7
FPD Conventional	2	2	4
Control Dark Brooder	3	1	4
Control Conventional	2	1	3
Column totals	10	8	18

One bird (control, dark brooder) was excluded from the analysis because of a very low number of observed OTUs (2), due to very few reads being produced for this sample during sequencing. Another two birds (one FPD dark brooder, one control conventional) were excluded as outliers due to extremely high Chao1 index values, suggesting that those samples contained a much higher abundance of rare OTUs than other samples, and therefore may not have been representative of the microbiome composition of the groups overall.

3.3.7. Statistical analysis

All statistical analysis was conducted in R Studio (R Core Team, 2023). All linear mixed models were conducted using the 'afex' package (Singmann *et al.*, 2023), and where significant interaction effects were found, post hoc pairwise comparisons were computed using the 'emmeans' package (Lenth, 2024). Firstly, a three-way ANOVA was used to analyse body mass with FPD status, rearing, and KBD as between-subject factors. An ANCOVA was used to analyse the mass of the brain, with body mass included as a continuous covariate.

A linear mixed model was used to analyse DCX^+ cell density, with FPD status, rearing, and KBD included as between-subject factors, and both cell type (bipolar vs multipolar) and subregion of HF (rostral vs caudal) were included as within-subject factors. The main effect of shed was also included in the model as an additional fixed factor. A linear mixed model was used to analyse effects of cumulative FPD score (treated as a continuous variable) on DCX^+ cell density. Rearing and KBD status were included as between-subject factors, and cell type and subregion of HF were included as within-subject factors. Again, the main effect of shed was included in the model as an additional fixed factor.

Alpha diversity in the caecal microbiome of each individual was measured using four metrics. These included observed taxa (simply the number of observed OTUs in each sample), Chao1 index (a measure of richness which gives more weight to rare taxa), and Shannon and Simpson indices, which account for both richness and evenness. Wilcoxon rank sum tests were used to analyse the effects of FPD, rearing, and KBD on these four metrics of alpha diversity between subjects. A Kruskal Wallis test was used to analyse the effect of housing sheds on the same four metrics of alpha diversity.

Principle coordinates analysis (PCoA) based on Jaccard distance was used to visualise the effects of FPD status, rearing, KBD, and housing sheds on beta diversity using the 'vegan' package in R (Oksanen *et al.*, 2022). A permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was conducted using the 'adonis2' function in order analyse the main effects of and interactions between the effects of FPD status, rearing, and KBD on beta diversity. A separate PERMANOVA with 999 permutations was conducted to analyse differences in beta diversity between housing sheds. FPD status and KBD were included in this model as covariates, though it was not possible to compute all interactions between the effects of shed, FPD and KBD due to an insufficient number of samples in some groups.

The relative abundances of taxa were calculated at phylum and genus level and were plotted for all taxa. OTUs belonging to phyla with median abundance > 0.01% were carried forward for analysis of differential abundance of OTUs between groups using the 'DESeq2' package (Love, Huber and Anders, 2014). Log_2 fold changes in the abundance of each OTU were compared between FPD vs control, dark brooder vs conventionally reared, and birds with vs without KBD. To account for multiple comparisons in this analysis, P values were automatically adjusted in DESeq2 with a correction for false discovery rate.

3.4. Results

3.4.1. Body and brain mass

FPD birds tended to be heavier than controls (Figure 3.2), though this did not reach the threshold for significance ($F_{1,13} = 3.84$, $p = 0.072$). The interaction effect between FPD status and rearing also did not reach the threshold for significance ($F_{1,13} = 3.91$, $p = 0.070$). In dark brooder reared birds, those with FPD tended to be heavier than controls, however in conventionally reared birds the body mass of those with and without FPD was very similar. There was also no significant main effect of rearing ($F_{1,13} = 0.40$, $p = 0.537$) or KBD ($F_{1,13} = 0.03$, $p = 0.877$), and no significant two-way interactions between the effects of FPD and KBD ($F_{1,13} = 0.23$, $p = 0.637$) or rearing and KBD ($F_{1,13} = 0.51$, $p = 0.486$). There was no three-way interaction between FPD status, rearing, and KBD ($F_{1,13} = 0.22$, $p = 0.654$).

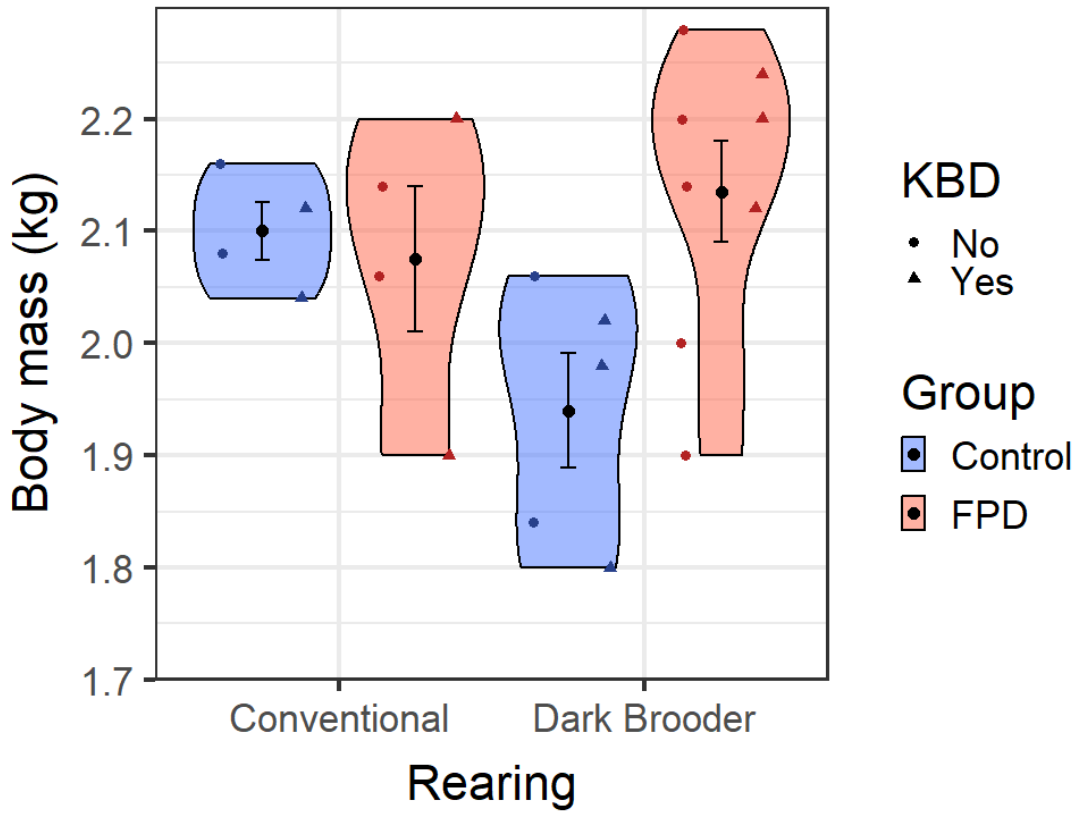

Figure 3.2 – Distributions of body mass (kg) at 70 weeks of age for control and FPD birds with and without KBD, reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE body mass.

Body mass did not predict brain mass ($F_{1,12} = 1.00$, $p = 0.338$). After correction for body mass, the interaction between the effects of FPD and rearing on whole brain mass was close to significance ($F_{1,12} = 4.60$, $p = 0.053$). Brain mass was very similar between FPD and controls in dark brooder reared birds, but conventionally reared FPD birds tend to have slightly heavier brains while conventionally reared control birds tend to have slightly lighter brains (Figure 3.3).

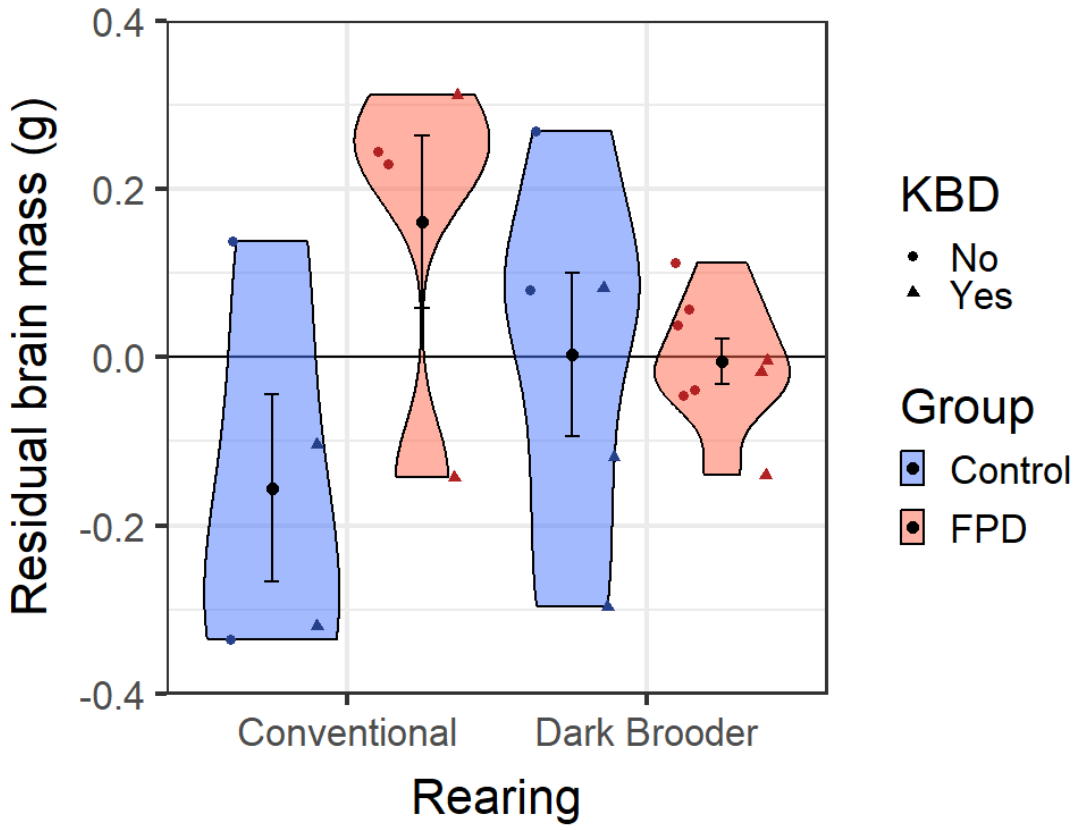

Figure 3.3 - Distributions of residual brain mass (after correction for body mass at 70 weeks of age) for control and FPD birds with or without KBD, reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE residual brain mass.

There were no significant main effects of FPD status ($F_{1,12}$ = 2.29, p = 0.156), rearing ($F_{1,12}$ = 0.10, p = 0.754), or KBD ($F_{1,12}$ = 3.84, p = 0.074) on brain mass, though birds with KBD tended to have lighter brains than birds without KBD. There were no significant two-way interactions between the effects of FPD and KBD ($F_{1,12}$ = 0.42, p = 0.532) or rearing and KBD ($F_{1,12}$ = 0.09, p = 0.772), and no three-way interaction between FPD status, rearing, and KBD ($F_{1,12}$ = 0.56, p = 0.469).

3.4.2. Effect of FPD status on DCX⁺ cell density

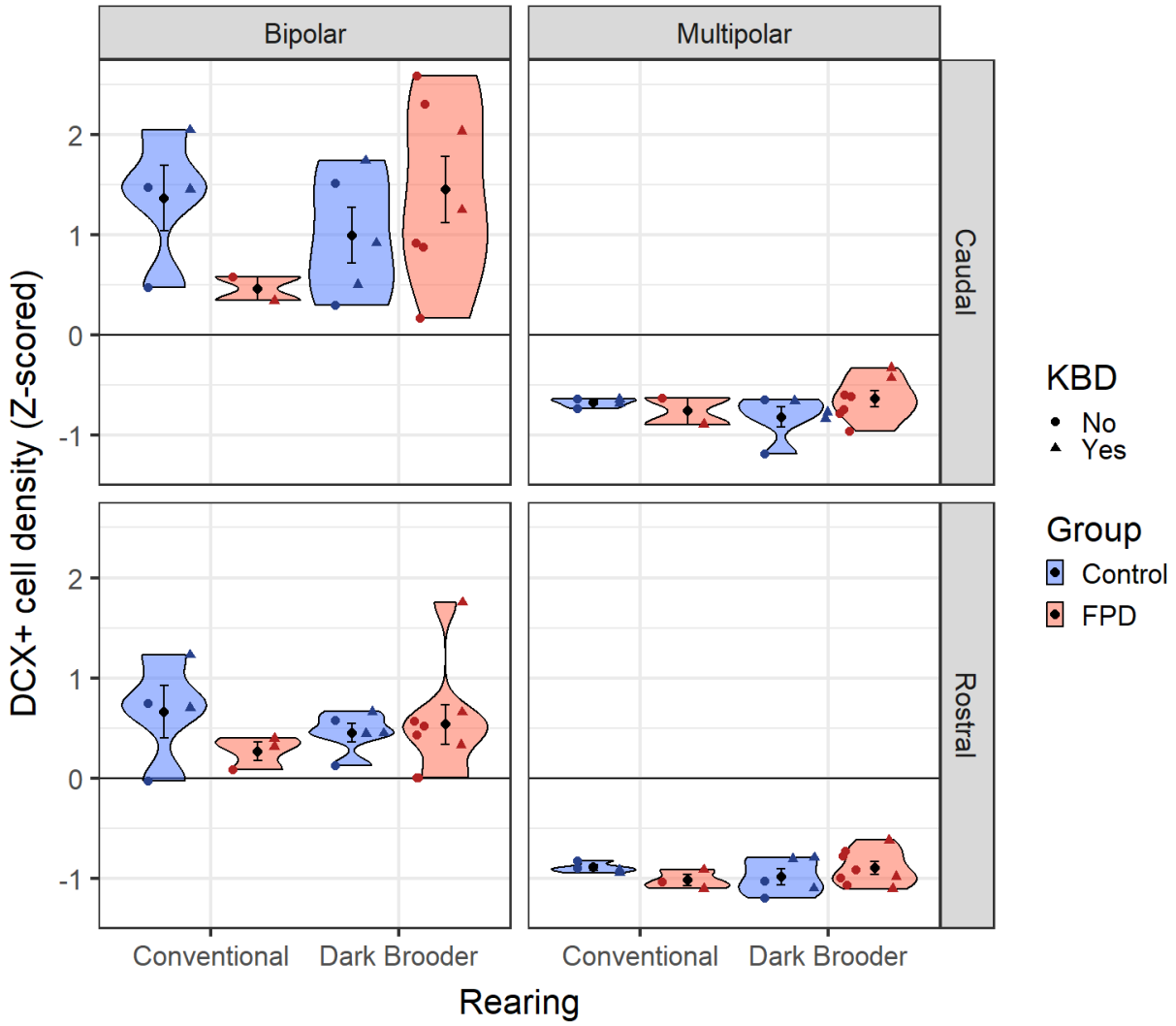

Figure 3.4 - Distributions of the densities of bipolar and multipolar DCX⁺ cells (Z-scored within staining batches) in the rostral and caudal HF of for control and FPD birds with or without KBD, reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE DCX⁺ cell density.

There was a significant interaction between the effects of FPD status and rearing on DCX⁺ cell density ($F_{1,40}$ = 4.23, p = 0.046). Post hoc pairwise comparisons show that while there was no significant difference in cell density between FPD and control birds that were reared with (p = 0.179) or without (p = 0.197) dark brooders, these effects were in opposite directions (Figure 3.4). FPD tended to decrease DCX⁺ cell density across the HF in conventionally reared birds, while it tended to increase DCX⁺ cell density across the HF in birds reared with a dark brooder. There was no significant effect of rearing on DCX⁺ cell density in control birds (p = 0.532), or FPD birds, though dark brooder reared FPD birds tended to have higher DCX⁺ cell density than FPD birds that were reared conventionally (p = 0.063).

Table 3.4 - Results from linear mixed model of the effects of FPD status, rearing, KBD, hippocampal subregion, cell type and shed on DCX+ cell density. The "Significance" column indicates the level of significance of each result using the following codes: non-significant (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Effect	Df	F	*p*	Significance
FPD status	1,40	0.04	0.836	ns
Rearing	1,40	2.56	0.118	ns
KBD	1,40	4.58	0.038	*
Subregion	1,40	13.59	< 0.001	***
Cell type	1,40	197.92	< 0.001	***
Shed	4,40	1.67	0.176	ns
FPD status*Rearing	1,40	4.23	0.046	*
FPD status*KBD	1,40	0.46	0.500	ns
Rearing*KBD	1,40	0.03	0.857	ns
FPD status*Subregion	1,40	0.01	0.907	ns
Rearing*Subregion	1,40	0.33	0.571	ns
KBD*Subregion	1,40	0.10	0.749	ns
FPD status*Cell type	1,40	0.78	0.383	ns
Rearing*Cell type	1,40	0.72	0.402	ns
KBD*Cell type	1,40	1.40	0.244	ns
Subregion*Cell type	1,40	2.32	0.136	ns
FPD status*Rearing*KBD	1,40	0.00	0.981	ns
FPD status*Rearing*Subregion	1,40	1.05	0.312	ns
FPD status*KBD*Subregion	1,40	0.27	0.603	ns
Rearing*KBD*Subregion	1,40	0.08	0.779	ns
FPD status*Rearing*Cell type	1,40	2.48	0.123	ns
FPD status*KBD*Cell type	1,40	0.10	0.754	ns
Rearing*KBD*Cell type	1,40	0.46	0.501	ns
FPD status*Subregion*Cell type	1,40	0.12	0.729	ns
Rearing*Subregion*Cell type	1,40	0.26	0.611	ns
KBD*Subregion*Cell type	1,40	0.20	0.657	ns
FPD status*Rearing*KBD*Subregion	1,40	0.40	0.531	ns
FPD status*Rearing*KBD*Cell type	1,40	0.91	0.345	ns
FPD status*Rearing*Subregion*Cell type	1,40	0.56	0.459	ns
FPD status*KBD*Subregion*Cell type	1,40	0.31	0.580	ns
Rearing*KBD*Subregion*Cell type	1,40	0.09	0.772	ns
FPD status*Rearing*KBD*Subregion*Cell type	1,40	0.06	0.813	ns

Across the whole HF, the density of multipolar DCX$^+$ neurons was significantly lower than the density of bipolar DCX$^+$ neurons ($F_{1,40}$ = 197.92, p < 0.001). There was a significantly lower density of DCX$^+$ cells (across both cell types) in the rostral HF than in the caudal HF ($F_{1,40}$ = 13.59, p < 0.001). Across all other groups, DCX$^+$ cell density was significantly higher in birds with KBD than birds without KBD ($F_{1,40}$ = 4.58, p = 0.038). There were no other significant main effects or interactions. The full results from this analysis can be found in Table 3.4.

3.4.3. Effect of cumulative FPD score on DCX$^+$ cell density

There was a significant interaction between the effects of cumulative FPD score and rearing ($F_{1,40}$ = 4.41, p = 0.042) (Figure 3.5). Across all other treatments, the density of DCX$^+$ cells decreased as cumulative FPD score increased in conventionally reared birds. However, in dark brooder reared birds, DCX$^+$ cell density increased as cumulative FPD score increased. Across the whole HF, there was a significantly lower density of multipolar DCX$^+$ neurons than bipolar DCX$^+$ neurons ($F_{1,40}$ = 71.79, p < 0.001). There were no other significant main effects or interactions. The full results from this analysis can be found in Table 3.5.

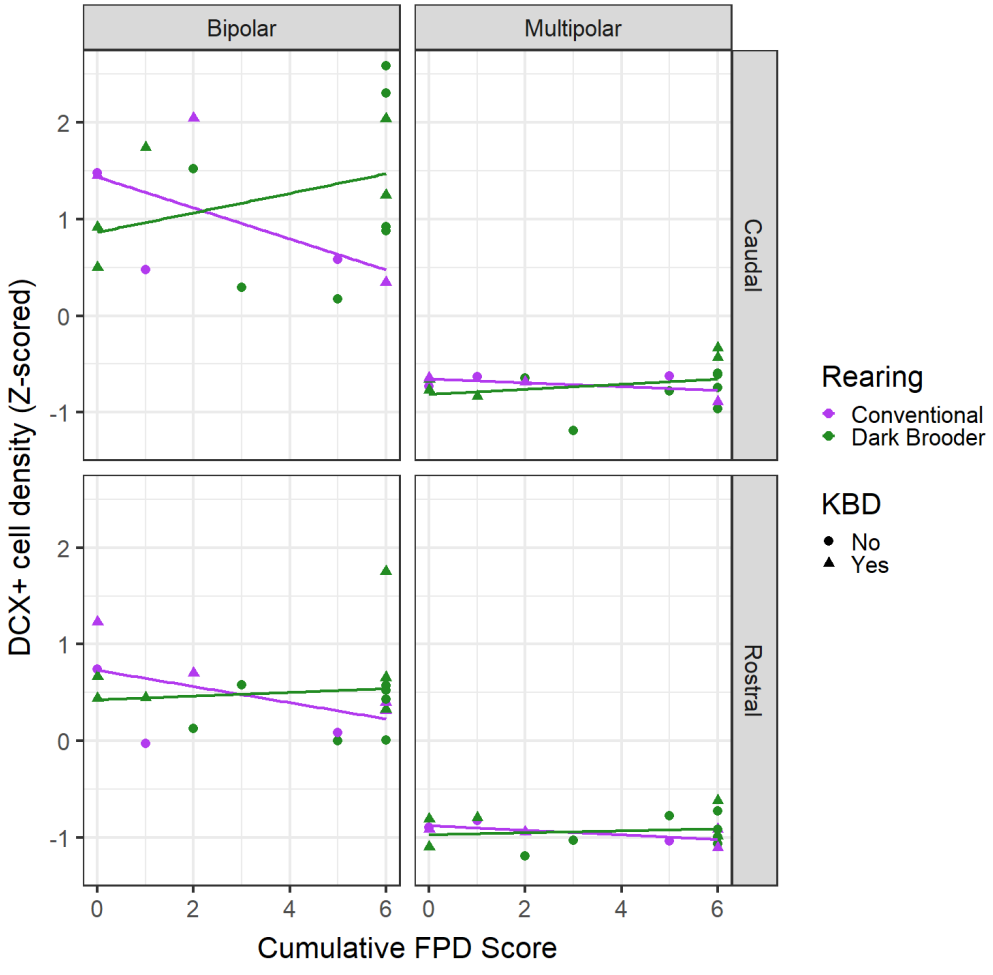

Figure 3.5 - Effects of cumulative FPD score, rearing, KBD, cell type and subregion of HF on DCX+ cell density (Z-scored).

Table 3.5 - Results from the linear mixed model of the effects of cumulative FPD score (Score), rearing, KBD, hippocampal subregion, cell type, and shed on DCX+ cell density. The "Significance" column indicates the level of significance of each result using the following codes: non-significant (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Effect	Df	F	*p*	Significance
Score	1,40	0.01	0.917	ns
Rearing	1,40	0.06	0.802	ns
KBD	1,40	0.78	0.384	ns
Subregion	1,40	3.30	0.077	ns
Cell type	1,40	71.79	< 0.001	***
Shed	4,40	1.61	0.191	ns
Score*Rearing	1,40	4.41	0.042	*
Score*KBD	1,40	0.29	0.593	ns
Rearing*KBD	1,40	0.04	0.836	ns
Score*Subregion	1,40	0.12	0.732	ns
Rearing*Subregion	1,40	0.21	0.649	ns
KBD*Subregion	1,40	0.05	0.821	ns
Score*Cell type	1,40	0.45	0.505	ns
Rearing*Cell type	1,40	0.95	0.336	ns
KBD*Cell type	1,40	1.18	0.284	ns
Subregion*Cell type	1,40	0.58	0.451	ns
Score*Rearing*KBD	1,40	0.02	0.902	ns
Score*Rearing*Subregion	1,40	0.73	0.398	ns
Score*KBD*Subregion	1,40	0.14	0.715	ns
Rearing*KBD*Subregion	1,40	0.00	0.951	ns
Score*Rearing*Cell type	1,40	3.00	0.091	ns
Score*KBD*Cell type	1,40	0.03	0.855	ns
Rearing*KBD*Cell type	1,40	0.54	0.466	ns
Score*Subregion*Cell type	1,40	0.00	0.973	ns
Rearing*Subregion*Cell type	1,40	0.18	0.675	ns
KBD*Subregion*Cell type	1,40	0.05	0.827	ns
Score*Rearing*KBD*Subregion	1,40	0.10	0.755	ns
Score*Rearing*KBD*Cell type	1,40	0.12	0.726	ns
Score*Rearing*Subregion*Cell type	1,40	0.58	0.449	ns
Score*KBD*Subregion*Cell type	1,40	0.14	0.707	ns
Rearing*KBD*Subregion*Cell type	1,40	0.07	0.786	ns
Score*Rearing*KBD*Subregion*Cell type	1,40	0.26	0.615	ns

3.4.4. Alpha diversity in the caecal microbiome

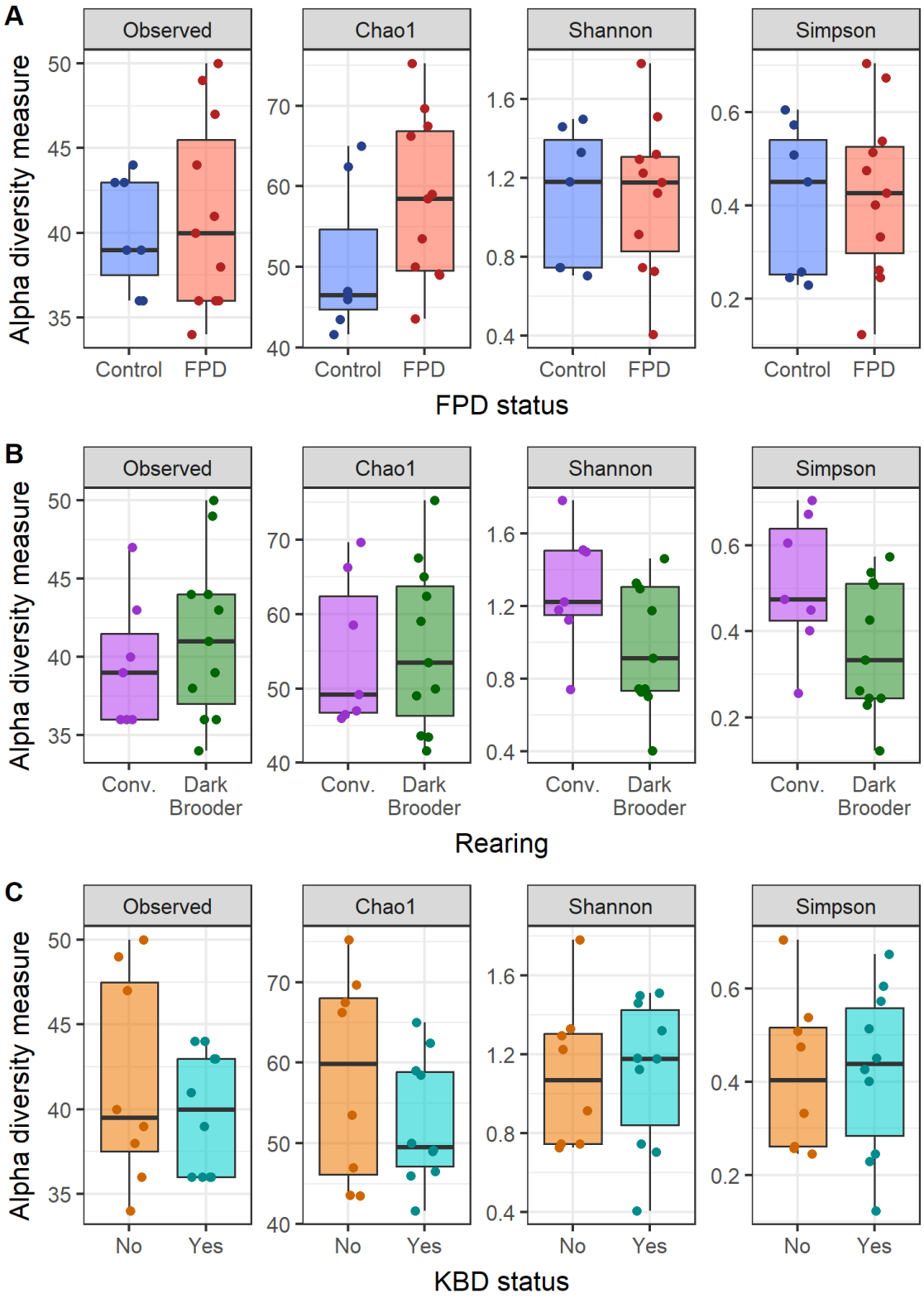

*Figure 3.6 – Boxplots showing distribution of alpha diversity measured by four metrics (observed taxa, Chao1, Shannon, Simpson) in **A)** FPD vs control birds, **B)** conventionally reared vs dark brooder reared birds, and **C)** birds with (Yes) vs without (No) KBD. Black horizontal bars indicate the median value for each alpha diversity metric in each group. Points represent individual samples.*

The difference in Chao1 index between FPD and control birds was approaching significance (W = 17.0, p = 0.057), however there were no significant effects of FPD, rearing, or KBD on any alpha diversity metrics (Figure 3.6). Full results of the Wilcoxon rank sum tests are presented in Table 3.6.

Table 3.6 - Test statistic (W) and P values from Wilcoxon rank sum tests on for the effects of FPD, rearing, and KBD on four metrics of alpha diversity (observed taxa, Chao1, Shannon, and Simpson).

		FPD		**Rearing**		**KBD**	
		W	***p***	**W**	***p***	**W**	***p***
Alpha diversity metric	**Observed**	36.5	0.891	31.0	0.521	45.5	0.653
	Chao1	17.0	0.057	38.0	1.000	51.0	0.351
	Shannon	39.0	1.000	56.0	0.124	38.0	0.894
	Simpson	36.0	0.856	57.0	0.103	40.0	1.000

The median number of observed OTUs tended to be higher in sheds C2 and DB1 than sheds C1 and DB2 (Figure 3.7), however, the difference in observed OTUs between sheds was not significant (χ^2_3 = 7.03, p = 0.071). There was no significant shed effect on alpha diversity as measured by the Chao1 (χ^2_3 = 1.65, p = 0.648), Shannon (χ^2_3 = 4.48, p = 0.214), or Simpson (χ^2_3 = 4.70, p = 0.195) indices.

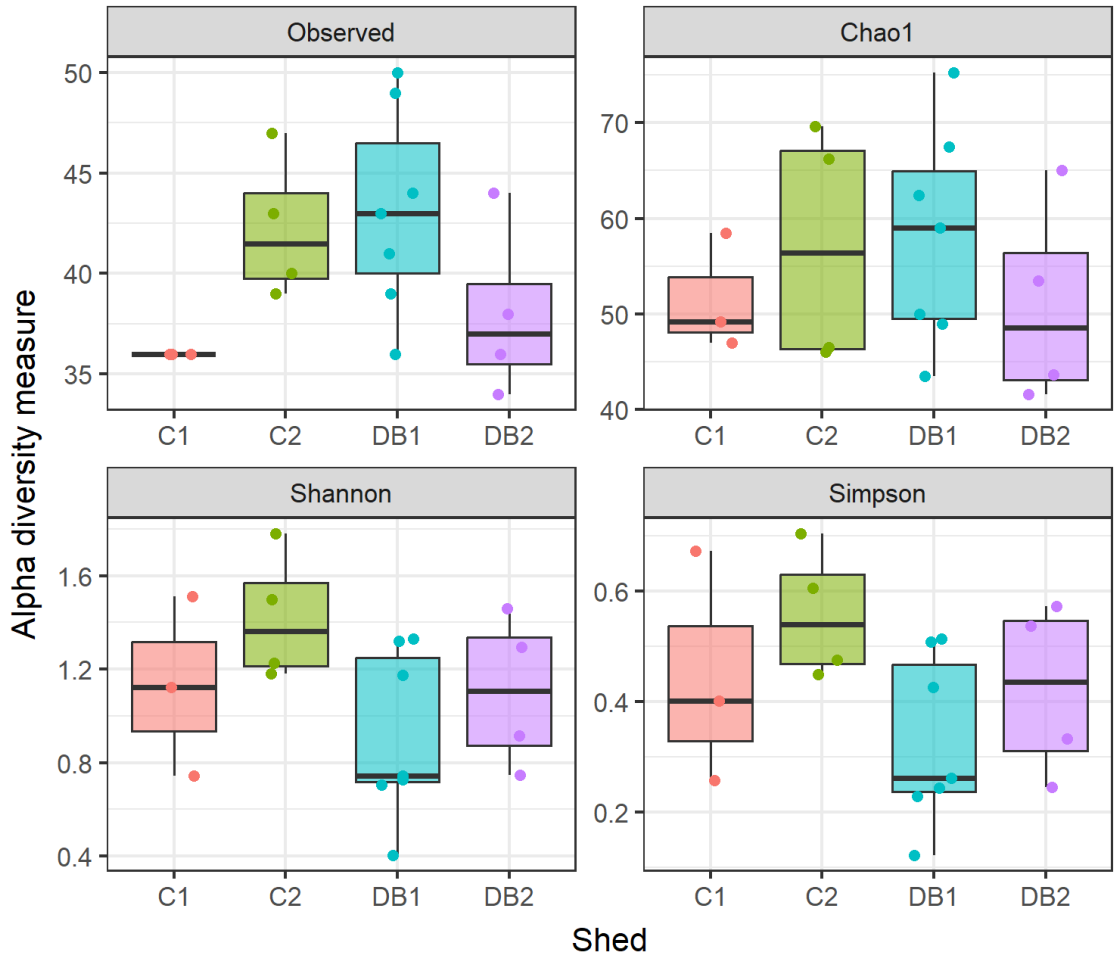

Figure 3.7 - Boxplots showing distribution of alpha diversity measured by four metrics (observed taxa, Chao1, Shannon, Simpson) in four sheds which housed birds which were reared conventionally (C1 and C2) or with a dark brooder (DB1 and DB2).

3.4.5. Beta diversity in the caecal microbiome

There was no main effect of FPD ($F_{1,10}$ = 1.23, p = 0.281), rearing ($F_{1,10}$ = 1.10, p = 0.315), or KBD ($F_{1,10}$ = 0.58, p = 0.742) on beta diversity (Figure 3.8). There were also no two-way interactions between FPD and rearing ($F_{1,10}$ = 1.38, p = 0.205), FPD and KBD ($F_{1,10}$ = 0.54, p = 0.779), or rearing and KBD ($F_{1,10}$ = 0.58, p = 0.732), and there was no three-way interaction between the effects of FPD, rearing and KBD on beta diversity ($F_{1,10}$ = 1.50, p = 0.212).

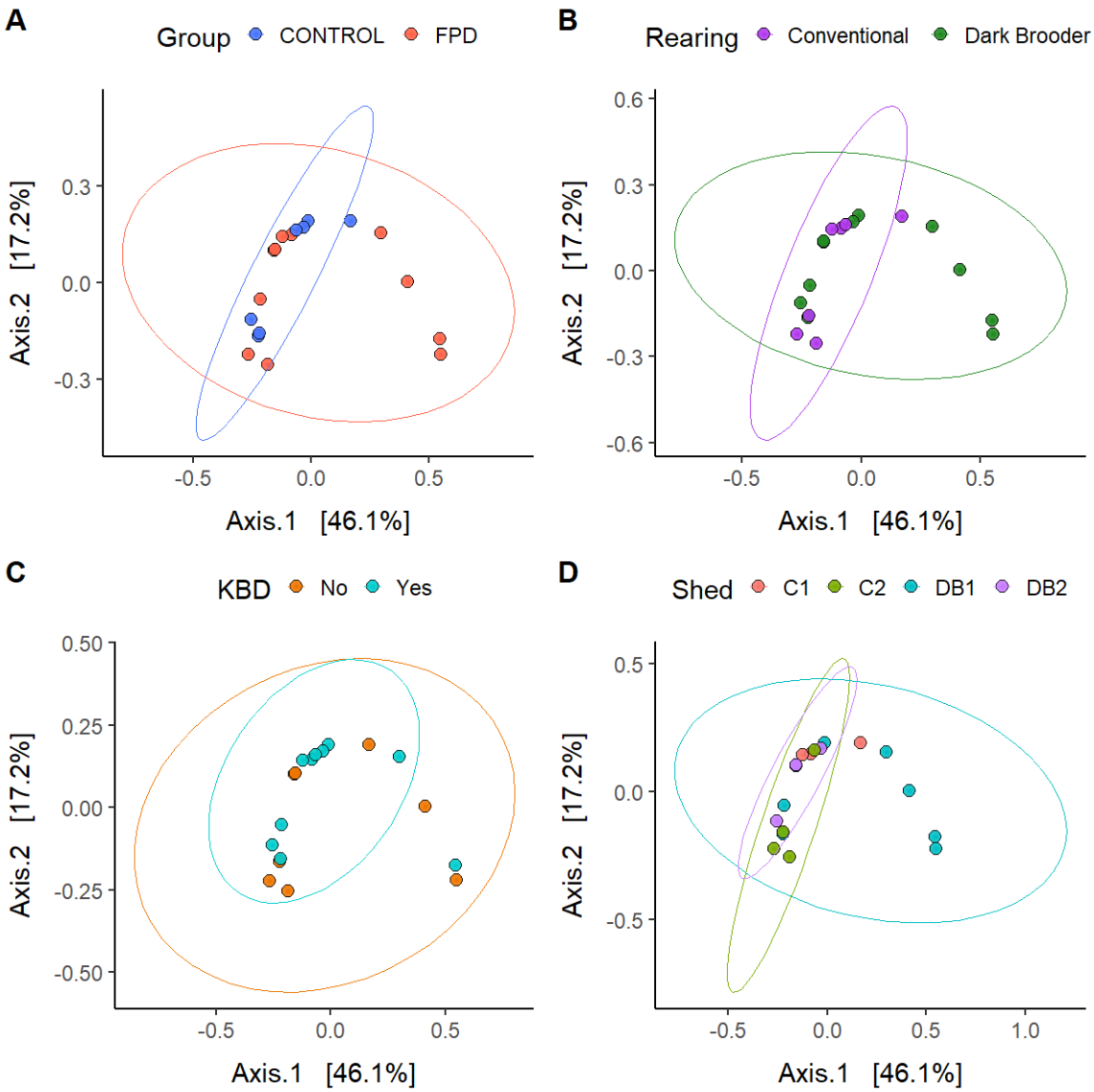

*Figure 3.8 – PCoA plots using Jaccard distance in order to visualise differences in beta diversity between populations of **A)** FPD vs control birds, **B)** conventionally reared vs dark brooder reared birds, **C)** birds with (Yes) vs without (No) KBD, and **D)** birds housed in four sheds (C1, C2, DB1, DB2). Ellipses show 95% confidence intervals for the cluster of individuals in each group. It was not possible to calculate an ellipse for shed C1 due to an insufficient number of samples.*

When controlling for the effects of FPD and KBD, the difference in beta diversity between the sheds did not reach the threshold for significance ($F_{3,12}$ = 1.87, p = 0.052). There was still no main effect of FPD status ($F_{1,10}$ = 1.00, p = 0.382) or KBD ($F_{1,10}$ = 0.61, p = 0.711).

3.4.6. Taxa abundance in the caecal microbiome

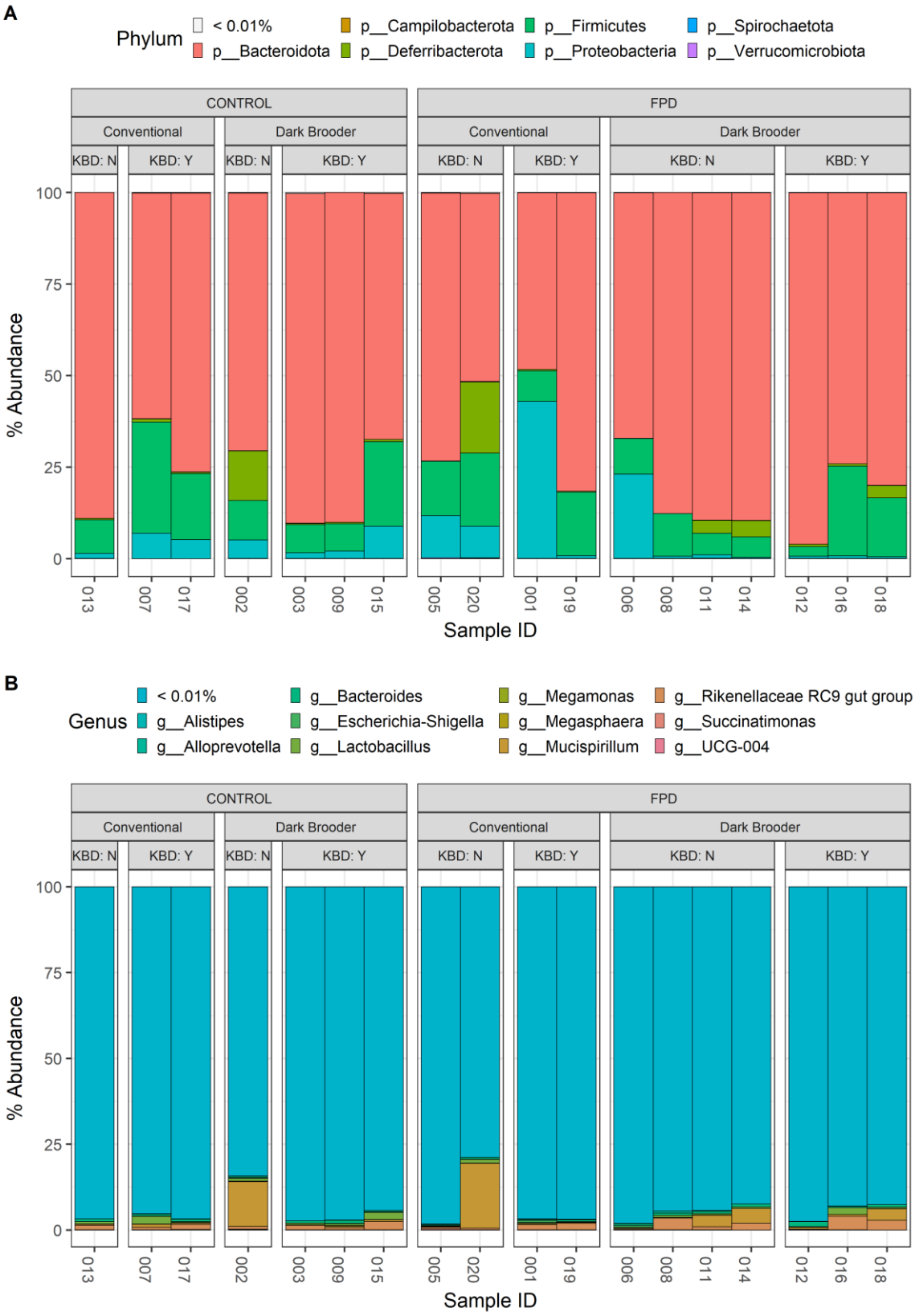

*Figure 3.9 – Relative abundance of taxa in each sample displayed at **A)** phylum and **B)** genus levels for all taxa with a median abundance > 0.01%. Taxa with median abundance < 0.01% are displayed as a single group. Individual samples are arranged by FPD status, rearing, and KBD status.*

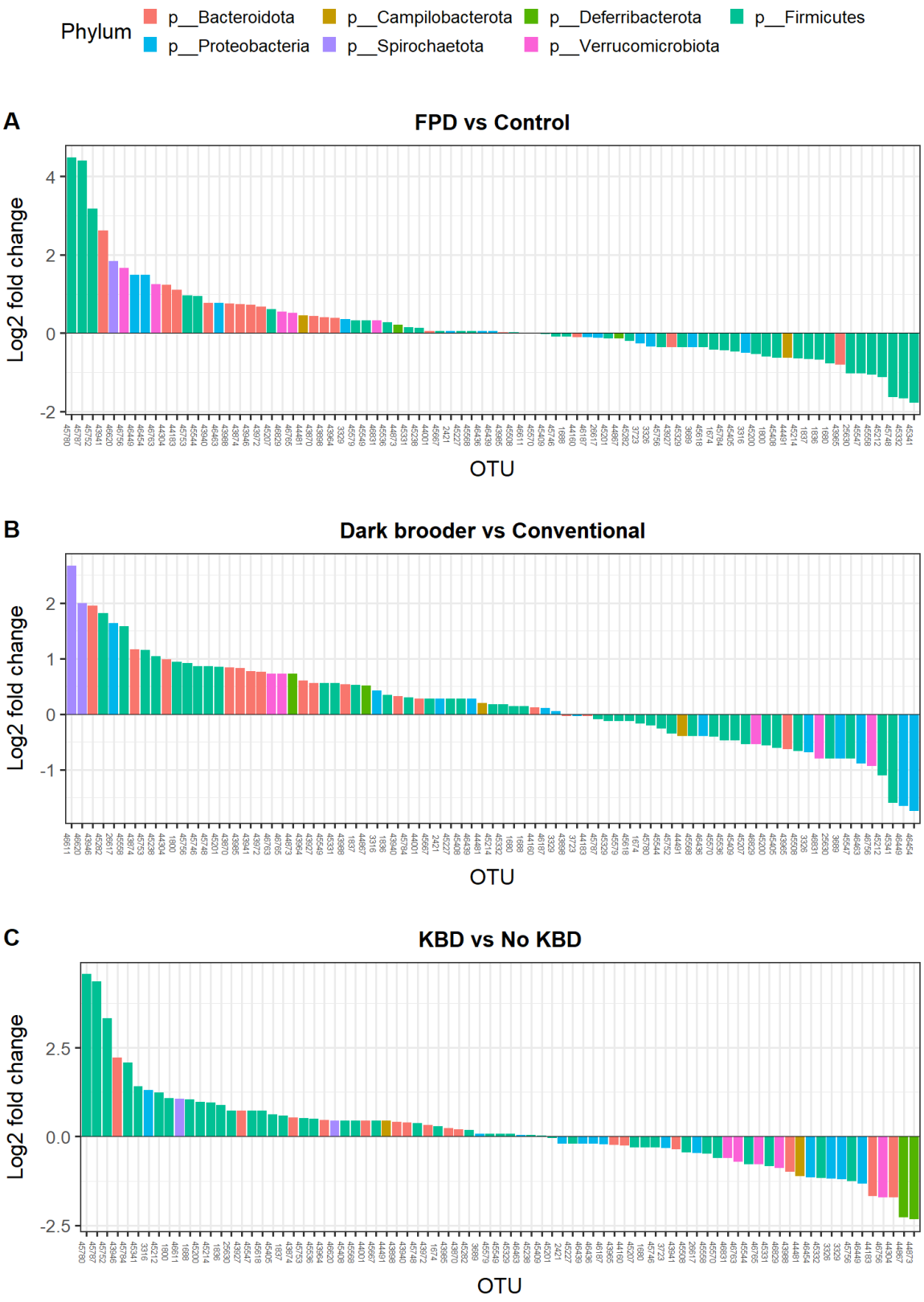

*Figure 3.10 - Differential abundance of OTUs (log_2 fold change from base mean) between **A)** FPD vs control birds, **B)** dark brooder reared vs conventionally reared birds, and **C)** birds with vs without KBD. OTUs are ranked in order of log_2 fold change, with positive values indicating greater abundance of OTUs in FPD, dark brooder reared, or KBD birds. Negative values indicate a greater abundance of OTUs in control or conventionally reared birds, or those without KBD. Coloured bars indicate the phylum to which each OTU belongs.*

There were seven phyla with a median relative abundance > 0.01% (Figure 3.9A). Across all groups, the most abundant phylum in the caecal microbiome of all individuals was Bacteroidota (median abundance 78%). There was also a relatively high abundance of Firmicutes (median abundance 11.2%) and Proteobacteria (median abundance 1.75%), with the caecal microbiome in one individual comprised of 42.6% Proteobacteria. All other phyla had a median relative abundance < 1%, though two individuals had a high abundance of Deferribacterota (19.4% and 13.5%), mostly of the genus *Mucispirillum* which had a median relative abundance of 18.8% and 13.1% in these samples respectively.

The vast majority of the caecal microbiome was comprised of OTUs which each had a median abundance of < 0.01%. When treated a single group, these rare genera had a median relative abundance of 96.1% across all groups (Figure 3.9B). The most abundant genera were the *Rikenellaceae RC9 gut group* (median abundance 1.24%), *Bacteroides* (median abundance 0.63%), *Mucispirillum* (median abundance 0.50%), and *Lactobacillus* (median abundance 0.49%). All other genera had a median abundance < 0.05%.

There were only two OTUs which were differentially abundant between FPD and control birds (Figure 3.10A). These were both Firmicutes of the family Veillonellaceae, but of unknown genus, and were more abundant in FPD birds than controls (test statistic = 3.85, adjusted p = 0.011; test statistic = 3.57, adjusted p = 0.017 respectively). There tended to be a higher abundance of the genus *Alloprevotella* in FPD birds than controls, though this was not a significant difference (test statistic = 3.07, adjusted p = 0.066). There were no OTUs that were differentially more abundant in control birds than FPD birds. There were no OTUs which were differentially abundant between dark brooder and conventionally reared birds (Figure 3.10B).

There were two OTUs that were significantly more abundant in birds with KDB than with without KBD (test statistic = 4.22, adjusted p = 0.002; test statistic = 4.03, adjusted p = 0.003 respectively). These were the same two OTUs (Firmicutes, family Veillonellaceae) which were differentially more abundant in FPD birds than controls. There were no OTUs which were significantly more abundant in birds without KBD than birds with KBD (Figure 3.10C), though the two OTUs which had the largest increase (log_2 fold change) in abundance in birds without KBD were both from the phylum Deferribacterota, one of which was *Mucispirillum* (test statistic = -2.54, adjusted p = 0.277), and one of which the genus was unknown (test statistic = -2.52, adjusted p = 0.277).

3.5. Discussion

While plasticity tended to decrease in conventionally reared birds in response to FPD as expected, plasticity tended to increase in dark brooder reared birds in response to FPD. These results support the hypothesis that FPD is a chronic stressor in conventionally reared birds, and also support the hypothesis that dark brooders may confer resilience to stress caused by FPD. There was a significantly higher DCX^+ cell density in the caudal HF than the rostral HF, and an overall higher density of bipolar DCX^+ cells than multipolar DCX^+ cells, as expected.

Some OTUs of the family Veillonellaceae were differentially more abundant in the caecal microbiome of FPD birds compared to controls, and in birds with KBD compared to birds without KBD, but there were no OTUs which were differentially abundant between birds reared with or without a dark brooder. Also, there were no differences in distributions of alpha diversity or beta diversity in the caecal microbiome between groups. These results partially support the hypothesis that chronic stress caused by FPD changes the composition of the microbiome, but the effects were limited, and it is unclear whether the differences in abundant taxa were truly an effect of chronic stress or direct infection. The findings from the study are discussed in full below.

3.5.1. Body and brain mass

The lack of a main effect of FPD status on body mass suggests that any stress caused by FPD was not severe enough to significantly affect normal growth, though the trend towards FPD birds being heavier than controls was not expected. Increased body mass has been proposed as a risk factor for FPD because it may increase the area of contact between the footpads and the litter (Shepherd and Fairchild, 2010), so it may be possible that we are observing an effect of body mass on FPD severity rather than the effect of FPD on body mass. However, this does not explain the non-significant trend towards an interaction between the effects of FPD and rearing on body mass. Also, it is unclear why conventionally reared FPD birds tended to have slightly heavier brains than controls after correction for body mass. Perhaps it would be more appropriate to correct for body size using a measure such as tarsus length rather than body mass.

3.5.2. Effects of FPD are dependent on rearing

The interactions between FPD severity and rearing on DCX^+ cell density suggest that FPD was only a significant chronic stressor to birds that were reared without a dark brooder. Though the post hoc tests following the interaction effect between FPD status and rearing did not

show a significant effect of FPD in either rearing group, the effect of FPD was in the expected direction of effect in conventionally reared birds, supporting the hypothesis that FPD is a chronic stressor. The effect of FPD in the group reared with a dark brooder, though non-significant, is in the opposite direction to what was predicted, with increased DCX^+ density across the whole HF in response to severe FPD. This suggests that rearing changes the way the HF responds to chronic stress.

Similarly, there was an interaction between cumulative FPD score and rearing. The relationship between cumulative FPD score and hippocampal plasticity was in the predicted direction for conventionally reared birds, adding further support to the hypothesis that FPD is a chronic stressor. However, for dark brooder reared birds, hippocampal plasticity increased as cumulative FPD score increased. Figure 3.5 shows that this pattern was observed across both subregions of the HF and in both cell types, and the effect was most pronounced in bipolar cells in the caudal HF. This has been proposed as the region which is most sensitive to chronic stress, and is, therefore, the region where I would expect to see an effect of a chronic stressor on hippocampal plasticity. However, there were no three-way interactions between FPD, rearing, and subregion, which suggests that the effects were not unique to the caudal HF and the current study does not provide further support for proposed functional specialisation of the caudal HF.

Taken together, the results go beyond the predicted effect of dark brooders in diminishing the effects of chronic stress. Instead, the results suggest that dark brooder reared birds have a response to chronic stress, but the direction of the effect on hippocampal plasticity is reversed. This may suggest that access to a dark brooder in the first weeks of life confers resistance to adult hens, which is consistent with previous behavioural studies which suggest that rearing chicks with a dark brooder may decrease behaviours associated with fearfulness and chronic stress (Sirovnik and Riber, 2022). Also, the results of the current study add to the previously known effects of dark brooders on FPD, and suggest that in addition to reducing average FPD severity (as in de Jong *et al.*, 2022), dark brooder rearing improves welfare in individuals which do have severe FPD.

However, the mechanism by which dark brooders may confer resilience is unclear. If it is true that animals reared with dark brooders are more resilient to stress, this may be due to a lower overall density of DCX^+ neurons in the HF from an early age. This could mean that upregulation of neural plasticity is needed when dark brooder reared birds are challenged with a chronic

stressor, unlike birds reared conventionally which have already developed a more sensitive stress response and have a higher density of DCX^+ neurons in the HF. It may be that the relationship between chronic stress and hippocampal plasticity follows an inverted U curve, in which plasticity initially increases with chronic stress, then reaches a threshold at which hippocampal plasticity begins to decrease as is often observed in animals experiencing depression-like states.

This proposed mechanism is currently speculation, and there are a number of limitations to the current study which mean we must be tentative about the results. Firstly, there was a low number of birds included in the analysis of hippocampal plasticity. The low sample size was partially due to the chosen methodology, as it is impractical to quantify DCX in a large number of birds when counting cells manually. Nine birds per group (for FPD vs control) is close to the typical sample size that we would use for this type of study, however, also investigating the effects of rearing and KBD in a factorial analysis meant that there were very few birds for each combination of treatments (Table 3.2). Also, there were disproportionally fewer birds in some groups compared to others, for example fewer birds were reared conventionally (n=6) than were reared with a dark brooder (n=12). The sample size was also limited by the FPD statuses of available birds, because there were very few birds with no signs of FPD by 70 weeks of age. Some of the control birds in this study had previous incidences of FPD, or were exposed to other stressors such as KBD, so cannot be considered true controls and further study would be needed to confirm these findings.

Regardless of the limitations, there is rationale for further studies on dark brooders in order to further investigate their effects on stress resilience. The effects of FPD were weak, and in order to design a more robust experiment investigating the effects of dark brooders on stress resilience, a well validated chronic stressor must be used instead. Also, we need to know the mechanism by which dark brooders may confer resilience to stress, which cannot be inferred from the design of the current study.

3.5.3. Effects of KBD on DCX^+ cell density

The direction of the main effect of KBD was unexpected, because increased severity of KBD has previously been shown to decrease hippocampal plasticity in hens (Armstrong *et al.*, 2020a). The increased DCX^+ cell density in KBD birds in the current study was only reported from the analysis in which I treated FPD and control birds as separate groups, and not in the analysis in which I treated cumulative FPD score as continuous, in which there was no

significant effect of KBD. The reason for this discrepancy between the two analyses is unclear, and cannot be easily explained by other factors in the study because there were no significant interactions between KBD and FPD or rearing.

Admittedly, KBD was not an original part of the experimental design and was only included in statistical models because it was a possible confounding variable which needed to be accounted for. The control birds included in the analysis of hippocampal plasticity were almost evenly balanced between those with (n = 5) and without KBD (n = 4), however, of the nine FPD birds included in that analysis, only three had KBD (Table 3.2). Especially with the small sample sizes in this study, the uneven distribution of KBD birds between the groups could have confounded the effect of FPD.

Ideally, all birds should have palpated for KBD before selection to eliminate KBD as a source of unwanted variation, however in such a small flock with a limited number of birds that could be considered true controls (in terms of FPD status), it may not have been possible to select a sufficient number of birds without KBD. Gebhardt-Henrich and Fröhlich (2015) found that all birds in their study which had severe FPD on both feet had a keel bone fracture at the end of life, and they suggest that foot injuries may increase the likelihood of falls due to inability to grip perches, subsequently increasing the likelihood of KBD. Therefore, it may have been difficult to find many FPD birds with no KBD even if sampling from a larger commercial flock.

3.5.4. Caecal microbiome

Alpha diversity distributions were not significantly different between any groups, according to any of the four metrics of alpha diversity. The two sheds in which birds had a tendency towards a higher number of observed taxa contained birds reared in different environments. This suggests that the non-significant trend towards a shed effect was driven by adult housing rather than the presence or absence of a dark brooder, which was not expected due to the importance of the rearing period for colonisation of the gut by bacteria.

The PCoA and PERMANOVA were based on Jaccard distance, which calculates distance between samples based on the presence or absence of taxa. Therefore, the results suggest that statistically, the same taxa were present across all groups. Though FPD had an effect on DCX^+ cell density (dependent on rearing), suggesting it is a chronic stressor, there was no main effect of FPD and no interaction between the effects of FPD and rearing on beta diversity, meaning the current study did not find evidence of a relationship between the composition of the caecal microbiome and chronic stress. Previously, DCX^+ cell density across the HF did not

significantly predict beta diversity, even though there was a significant effect of body condition on DCX^+ cell density (Armstrong *et al.*, 2022). It may be that hippocampal plasticity is more sensitive to the effects of chronic stressors such as poor body condition and FPD than caecal beta diversity. The difference in beta diversity between the sheds was close to significance, suggesting that in the current study, the adult housing or social group may have had a greater effect on caecal microbiome composition than the rearing environment, or any other effect that was tested.

Bacteroidota, Firmicutes, and Proteobacteria were the most abundant phyla overall in the current study, which is consistent with previous analyses of the caecal microbiome in laying hens (Shi *et al.*, 2019). Bacteroidota and Firmicutes are typically the most abundant phyla in the healthy chicken microbiome with around 45% relative abundance each (Rychlik, 2020), though in the current study two phyla were not equally abundant as Bacteroidota had a much higher median relative abundance of 78%. The overall high abundance of Proteobacteria is notable because some species of Proteobacteria, including *Salmonella spp.* and *E. coli* are common factors in human disease (Rizzatti *et al.*, 2017). The lack of difference in the relative abundance of Proteobacteria OTUs between groups may indicate that FPD, rearing, and KBD have minimal effects on food safety, though many other phyla are also involved in human disease and classification at species level would be needed to accurately quantify the pathogens present in each group.

The two OTUs that were differentially more abundant in FPD birds than control were both from the family Veillonellaceae, which has known functions associated with the digestion of fibre and production of the short chain fatty acids acetate and propionate (Tilocca *et al.*, 2016; Lecomte *et al.*, 2015). Short chain fatty acids are among bacterial metabolites which are involved in signalling in the gut-brain axis (Foster, Rinaman and Cryan, 2017), and are believed to have a role in regulating the stress response. There was a non-significant trend towards higher abundance of *Alloprevotella* in FPD birds compared to controls. *Alloprevotella* also has a known function in metabolising fibre to produce short chain fatty acids (Sun, Hou and Yang, 2021).

No taxa were differentially abundant between dark brooder and conventionally reared birds, which is consistent with the results of alpha and beta diversity suggesting that rearing did not affect the composition of the caecal microbiome. Perhaps this lack of difference was due to all birds being reared in the same large shed, and the effect of the presence of a dark brooder

was not great enough to cause differentially abundant gut bacteria to those reared with conventionally. Species which colonise the gut during early life persist in the adult gut, so it is not surprising that birds reared in the same shed have similar caecal microbiota. However, this conflicts with a previous study in broilers, in which dark brooder rearing affected the composition of microbiota in faecal samples, with higher *Lactobacillus* in birds reared with a dark brooder than in those reared conventionally (de Jong *et al.*, 2022). Other types of enrichment during rearing have also changed the caecal microbiome in layer pullets reared in same facility (Bari *et al.*, 2022).

The OTUs which were differentially more abundant in those with KBD compared to those without KBD were the same OTUs that were differentially more abundant in FPD birds compared to controls. FPD and KBD are both putative chronic stressors and it is interesting that they were both associated with higher abundance of Veillonellaceae.

The two individuals with high *Mucispirillum abundance* (one control dark brooder, one FPD conventional) were both from the group without KBD, and are likely to have made a large contribution to the trend towards higher Deferribacterota abundance in birds without KBD. Though the sequencing in the current study was only at genus level, here, a specific species can be identified because *Mucispirillum* is represented by the single species *M. schaedleri*, which has been associated with healthy development of the mucus layer in the intestinal tract of chickens (Varriale *et al.*, 2022). Also, *M. schaedleri* was found to colonise the gut of birds only after they were allowed to use an outdoor range (Varriale *et al.*, 2022), so may be related to range use. It is possible that birds with no KBD may be more likely to use the range. *Mucispirillum* was not found to be differentially abundant between birds with and without KBD in the current study, but this and Deferribacterota more widely, may be taxa of interest in future studies involving KBD.

Though I have attempted to interpret the differences in taxa abundance between groups, the results of the current study do not provide true functional information about the identified features of the caecal microbiome, and further study would be needed to investigate this. It is difficult for genus-level 16S rRNA sequencing to provide functional information, which is important for interpretation of results because two species from the same genus could have very different functions. To illustrate this, Schloss and Westcott (2011) use the example of the genus *Pseudomonas*, which could indicate the presence of either a commensal species or a pathogenic species, though it is impossible to know which of these is present using OTU-based

16S sequencing. Therefore, higher resolution species level sequencing is needed to accurately interpret the functions of differentially abundant taxa.

The OTU table used in the analysis of the caecal microbiome contained a lot of OTUs of which were not identified at genus level. Problems with taxa identification can arise from a lack of identified sequences stored in databases, a high abundance of novel species, or multiple species having similar or identical 16S rRNA sequences (Janda and Abbott, 2007). It is possible that some of the unknown OTUs could be from genera that are already identified separately, because multiple OTUs may belong to the same genus (Schloss and Westcott, 2011). Some of the unidentified OTUs may have belonged to important genera which may have been incorrectly sorted into the < 0.01% abundance category. It is possible that the abundances of some genera are higher than suggested in Figure 3.9B.

As with other areas of the current study, there was a low number of birds in each group, as low as one bird for some combinations of treatments (Table 3.3). Low sample sizes are typical in studies on the chicken microbiome. In a systematic review of the recent literature, Pires *et al.* (2024) reported sample sizes ranging between 4 and 20 samples per group, and suggested that much larger sample sizes (as are typically used in microbiome studies in humans) would be needed to accurately detect differences in the microbiome composition.

Interactions between the host genotype and gut microbiota are complex, and there are differences in gut microbiota composition between breeds, even when hatched and housed under the same climate-controlled conditions (Richards-Rios *et al.*, 2020a). Only one breed of hen was used in the current study. This is not necessarily a problem, because it means that breed was controlled across all treatment groups, but it would be ideal to investigate breed differences because it is possible that breed-dependent effects of FPD and dark brooding could exist.

3.5.5. Conclusions

In conclusion, the current study found support for the hypothesis that FPD is a chronic stressor, but only in birds reared without a dark brooder, as the effect of FPD on hippocampal plasticity was dependent on the condition in which birds were reared. While statistical power was low due to small sample sizes and the effects of FPD on hippocampal plasticity did not reach statistical significance when looking at each rearing condition separately, the opposite directions of the effects in each rearing condition are intriguing and call for further investigation of the effects of dark brooders on resilience to chronic stress.

Severe FPD and KBD birds both had increased abundance of taxa from the family Veillonellaceae in the caecal microbiome, which may have a role in regulating the stress response through production of short chain fatty acids. However, the information obtained from sequencing the microbiome was lacking true functional information about differentially abundant taxa, and the lack of differences in alpha and beta diversity suggest that differences in the composition of the caecal microbiome were minimal.

Chapter 4. Does early-life rearing environment affect stress resilience in laying hens?

4.1. Abstract

In conventional rearing systems, laying hen chicks do not have access to a mother hen. Some aspects of maternal care can be imitated using a dark brooder, which is a heating element with a curtain that provides warm and dark area in which birds can take refuge during the first few weeks of life. Though recent research has shown benefits to rearing with a dark brooder including reduced fearfulness and reduced injurious pecking, dark brooders are still not widely used on commercial rearing farms. In this study, I aimed to investigate whether rearing chicks with a dark brooder confers resilience to chronic stress later in life. Birds reared with or without a dark brooder were exposed to either an unpredictable chronic mild stress (UCMS) treatment or a control treatment for 8 weeks. In order to quantify the responses to the treatment, I measured duration in tonic immobility (TI), behaviours during novel object tests (NOT), blood plasma corticosterone (CORT) concentration, and hippocampal gene expression of three genes associated with plasticity (DCX, BDNF, and PCNA). I hypothesised that birds reared with a dark brooder would be more resilient to chronic stress than those reared conventionally.

TI durations were increased from a baseline only in UCMS birds after 6 weeks of treatment. This suggests that my UCMS protocol successfully induced chronic stress in the birds. There was a significant interaction between stress and time-point on time spent moving in the NOT. Dark brooder reared birds spent significantly more time close to the door of the testing arena than conventionally reared birds, and the interaction between the effects of rearing and circle in the NOT suggest that dark brooder birds may have been less anxious when exploring the areas of the arena closest to the novel object. Responses to acute stress in adulthood were dependent on both chronic stress and rearing condition, with elevated plasma CORT after restraint in a bag only observed in conventionally reared birds which were not exposed to UCMS. The expression of DCX at the caudal pole of the hippocampal formation (HF) provides my strongest support for the effects of dark brooder rearing on stress resilience in adulthood. In conventionally reared birds, UCMS caused slightly lower DCX expression in the caudal HF. However, in dark brooder birds, UCMS significantly increased DCX expression in the caudal HF, suggesting that adult responses to UCMS were dependent on the rearing environment. These results support the hypothesis that dark brooder rearing confers stress resilience, and

are consistent with the long term welfare benefits of dark brooders which have been reported in previous studies. Though the mechanisms by which dark brooder rearing may confer stress resilience are not fully understood, the results of this study add to call for the wider implementation of dark brooders on commercial rearing sites.

4.2. Introduction

Stress resilience is an important concept in animal welfare, which describes the ability of an animal to return to a baseline after a stressful event. This is distinct from the absence of a stress response (Bhatnagar, 2021), and the pattern of the return to baseline after a stressor can vary between individuals depending on their experiences, both in early life and adulthood. Anacker *et al.* (2018) has shown in mice that adult hippocampal neurogenesis, a form of hippocampal plasticity, confers resilience to chronic stress by mediating activity in the ventral hippocampus, the region which is most sensitive to stress in mammals. In previous chapters, I used hippocampal plasticity to measure the chronic stress experienced by hens using the density of cells expressing doublecortin (DCX) cell in the hippocampal formation (HF). Here, I measure the expression of DCX, brain-derived neurotrophic factor (BDNF), and proliferating cell nuclear antigen (PCNA) genes in the HF as markers of plasticity.

4.2.1. Chronic stress paradigms

In order to study stress resilience, chronic stress may be induced experimentally. Previous studies have employed many different methods to study the effects of chronic stress on animals. One method is to use chronic administration of corticosterone (CORT) to mimic chronic stress. For example, Pravosudov and Omanska (2005) implanted mountain chickadees with a pellet which released CORT over the course of 90 days. In that study, chronic CORT administration did not affect hippocampal volume or neurogenesis in the birds.

Many studies will induce chronic stress more directly by exposing the animals to stressful events over an extended period of time. A single stressor such as feed restriction (Robertson *et al.*, 2017) may be used over a long period of time to induce chronic stress. Similarly, An unpredictable feed schedule has also been used as chronic stressor in laying hens (Pusch *et al.*, 2018). In these birds, plasma CORT levels were elevated from baseline at day 7 of treatment, but returned to baseline by day 14 of treatment.

Unpredictability is a key component of many stress paradigms, especially unpredictable chronic mild stress (UCMS), in which an array of mild stressors are employed in an order that is not predictable by the study animals. Chronic mild stress paradigms evoke “an array of

neurobiological changes that mirror those seen in depression" (Hill *et al.*, 2012), making them useful for studies which in stress resilience is measured using a variety of physiological and behavioural outcomes. Dickens *et al.* (2009) induced chronic stress in wild-caught European starlings using a set of five acute stressors including loud and unfamiliar sounds, unpredictable movement, and restraint. These acute stressors were applied for 30 minutes at a time, in an unpredictable rotation 4 times a day for 16 days. Chronic stress significantly reduced glucocorticoid receptor (GR) expression in the hypothalamic paraventricular nucleus of starlings, but not in the hippocampus, while mineralocorticoid receptor (MR) expression was decreased in the hippocampus of chronically stressed starlings. Mild stress treatments may be applied as little as once per day in some studies, and are more typically conducted over a period of a few weeks, rather than exposing animals to multiple acute stressors per day for a shorter period of time as in Dickens *et al.* (2009). Most UCMS studies have been conducted in rodents, and UCMS is considered a well established model of depression in rats and mice and can be used to screen for appropriate candidates for antidepressant drug trials (Nollet, 2021), with antidepressant treatment reversing the effects of UCMS. For example, Willner *et al.* (1987) found that 5-9 weeks of a UCMS treatment decreased consumption of sucrose solution in rats, which is a common measure of anhedonia as an indicator of a depression. This effect was reversed by 2-4 weeks of treatment with an antidepressant drug (DMI). UCMS has also been found to increase coat score in mice, which is another indicator of a depression-like state (Farooq *et al.*, 2012).

Though not many UCMS studies have been conducted in birds, Gualtieri *et al.* (2019) showed that UCMS decreases the density of DCX^+ neurons at the caudal pole of the HF in chickens. In that study, the UCMS protocol consisted of 10 stressors, including changes in temperature or day length, food deprivation, removal of nest boxes, wet litter in the housing pens, artificial wind, chasing by humans, and social stressors such as isolation or mixing with unfamiliar conspecifics. In addition to these stressors, UCMS birds were exposed to an unpredictable lighting schedule each day. The UCMS treatment was conducted for 8 weeks, which was sufficient to elicit the observed changes in hippocampal plasticity. Gualtieri *et al.* (2019) also studied the effects of UCMS on blood plasma CORT, and found that while there was no difference in baseline CORT levels between UCMS and control birds after 2 weeks of treatments, there was higher baseline CORT in controls than UCMS birds after 7 weeks of treatments. In the current study, I used a similar UCMS protocol and also measured hippocampal plasticity (though by gene expression, not cell density) and plasma CORT (both

baseline and in response to an acute stressor). I aimed to investigate whether early-life experience affects the resilience of adult laying hens when they are challenged with a validated chronic stress paradigm as in (Gualtieri *et al.*, 2019).

4.2.2. Early-life effects on adult stress response

Again, most of the previous work on early-life effects on the adult stress response has been conducted in rodents. Rats lick or groom their offspring during early life, and this kind of maternal behaviour has region-specific effects on the hippocampus of adult rats (Nguyen *et al.*, 2015). Reduced maternal licking reduced long-term potentiation in dorsal hippocampus (which can impair memory) of adult rats compared to those whose mothers exhibited higher levels of licking, but reduced maternal licking increased long term potentiation in the ventral hippocampus of rats during adulthood. This increased activity in the ventral hippocampus, which is the region that is most sensitive to emotional stimuli, may lead to increased anxiety-related behaviours in adults (Felix-Ortiz *et al.*, 2013).

It has also been found that rat pups from mothers which exhibited more licking had lower adrenocorticotropic hormone (ACTH) and CORT responses to acute stress in adulthood (Liu *et al.*, 1997). This suggests that the effects of maternal behaviour during early life affect the developing HPA-axis of pups, thereby programming the adult stress response and conferring resilience to acute stress. Liu *et al.* (1997) suggest that this is early-life programming is adaptive, because it promotes an appropriate stress response in wild rats which are likely to experience a similar level of adversity during adulthood as they experience during early life, reducing the need for adaptation later in life. Nguyen *et al.* (2018) observed that after auditory fear conditioning, offspring of high-licking mothers exhibit more freezing behaviour in response to neutral stimuli compared to the offspring of lower-licking mothers. This suggests a more generalised fear response. This appears to conflict with the findings of Liu *et al.* (1997) and Nguyen *et al.* (2015), and suggests that in some contexts, early-life adversity (specifically, lower experience of positive maternal behaviours) can program more appropriate responses to adverse adult environments.

Early-life experiences have also been shown to have effects on the stress response which persist into adulthood in birds. Marasco *et al.* (2016) mimicked early-life adversity by injecting CORT (10µl 850 ng/ml) or a vehicle-only injection into the yolk of developing Japanese quail eggs on day 5 of incubation. Between days 5 and 19 after hatching, CORT was administered orally to 50% of the birds in each group, while the other 50% were controls. At 69-73 days of

age, birds were killed and samples of the hippocampus and hypothalamus were collected for analysis of gene expression. Compared to controls, birds treated with CORT during early life had upregulated expression of MR and BDNF in the hippocampus during adulthood. In the hypothalamus of adult birds, expression of genes including serotonin receptors (1D, 2C, 3A) and corticotropin releasing hormone receptor 2 (CRHR2) were upregulated only in birds which received the post-natal CORT administration. These findings show that early-life adversity can affect the adult stress response, and its effects depend on the developmental stage in which adversity is experienced.

Walker *et al.* (2019) also manipulated stress hormones in pre-natal Japanese quail, again by injecting exogenous CORT or a vehicle alone into the egg. After hatching, post-natal stress was induced in 50% of these birds from 4 days of age using an unpredictable feeding schedule for 15 days. Pre-natal stress increased expression of IL-1β (pro-inflammatory cytokine) in the pituitary gland at 246.5 days of age (mean age at tissue collection). Post-natal stress increased expression of IL-1β in pituitary gland, and decreased expression of colony-stimulating factor 1 receptor (CSF1R), a marker for microglia abundance, in the pituitary and hypothalamus, and reduced expression of IL-10 (anti-inflammatory cytokine) in the hypothalamus and amygdala. This provides evidence that stress during early life is involved in programming the HPA axis by moderating long-term expression of microglia and cytokines. The increased ratio of pro-inflammatory cytokines may affect responses to stress later in life.

4.2.3. Dark brooders

Unlike rat pups, laying hen chicks on commercial rearing farms do not have access to a mother hen. Dark brooders are an early-life intervention that mimic some aspects maternal care, and have been identified as a potential way to improve stress resilience in adult hens, though currently they are rarely used on commercial rearing farms. As discussed previously (Chapter 1 and Chapter 3), dark brooder rearing has been found to have welfare benefits including reduction of injurious pecking (Jensen, Palme and Forkman, 2006; Gilani, Knowles and Nicol, 2012; Sirovnik and Riber, 2022) and greater motivation for social reinstatement (de Jong *et al.*, 2022). In addition to the behavioural benefits of dark brooder rearing, Nordquist *et al.* (2020) found no difference in feather CORT between birds reared with and without a dark brooder. While this result does not add weight to the argument that dark brooders provide positive welfare, it suggests that they do not cause stress. Rearing with a broody hen has been found to affect lateralisation of cell soma size (visualised using Nissl staining) in the HF of hens

at 52 weeks of age, though there was no significant difference in average cell soma size between birds reared with or without a hen (Nordquist *et al.*, 2013). This suggests that brain-based measures of welfare may be useful for studying the effects of maternal care on the welfare of hens. Nordquist *et al.* (2020) measured the expression of arginine vasotocin (AVT) in the hypothalamus of birds reared with or without dark brooders. Using dark brooders tended to increase AVT^+ cell counts in the anteromedial preoptic nucleus and supraoptic nucleus at 16 weeks of age and 28 weeks of age, though similar trends were not observed in other areas of the hypothalamus. To my knowledge, there are no previous studies investigating effects of dark brooders on hippocampal plasticity specifically, and this may add to our understanding of how dark brooders may affect stress resilience in laying hens.

While plasticity can be used to measure resilience to chronic stress, it is also important to consider the effects of acute stress, and the interactions between the effects of chronic and acute stress are important when studying resilience and interventions which may confer resilience. Therefore, I also measured CORT as the output of the hypothalamic-pituitary-adrenal (HPA) axis in response to chronic and acute stressors. The effects of adult chronic stress on responses to acute stress were previously studied in starlings (Rich and Romero, 2005), and both baseline CORT and CORT elevation in response to acute stress were significantly reduced when birds were chronically stressed. In addition to physiological measures of stress resilience, I investigated anxiety related behaviours of laying hens, which were exposed to a chronic stress treatment.

4.2.4. Aims and hypotheses

In this study, I aimed to investigate the effects of rearing with a dark brooder on responses to chronic stress later in life by inducing chronic stress experimentally in conventionally reared and dark brooder reared laying hens using an unpredictable chronic mild stress (UCMS) protocol similar to that used by Gualtieri *et al.* (2019). In addition to studying the hippocampal plasticity as in previous chapters (here, gene expression related to hippocampal plasticity), I measured the response of the HPA axis to acute and chronic stress using blood plasma CORT titres. I also conducted behavioural tests: novel object tests (NOTs) and tonic immobility (TI).

It was hypothesised that birds reared with a dark brooder would be more resilient to chronic stress during adulthood than those reared conventionally. If this is true, I predict the following outcomes:

1. In conventionally reared birds, UCMS will decrease the expression of the three plasticity genes in the caudal hippocampus, which is known to be sensitive to chronic stress. In dark brooder reared birds, this decrease will be smaller or non-existent. I do not predict a significant decrease in the expression of plasticity related genes the rostral hippocampus.
2. In conventionally reared birds, UCMS will cause a decrease in baseline plasma corticosterone concentration compared to control birds. In dark brooder reared birds, this decrease will be smaller or non-existent.
3. After an acute-stress challenge, there will be a greater increase in plasma corticosterone from baseline in conventionally reared birds than in dark brooder reared birds.
4. In conventionally reared birds, UCMS will cause the following differences in behaviours during the NOTs. These differences will be smaller or non-existent in dark brooder reared birds.
    a. Greater latency to first movement
    b. Less time moving
    c. More time still
    d. More time freezing
    e. More time spent near the door of the arena
    f. Less time spent closer to the novel object
5. In conventionally reared birds, UCMS will cause an increased duration of TI. This increase will be smaller or non-existent in dark brooder reared birds.

This study was preregistered with the Open Science Framework (https://osf.io/tajme). This chapter also includes some exploratory elements that were not included in the preregistration. In addition to the preregistered hypotheses and predictions above, I also predicted that UCMS would decrease egg production, body mass, and spleen mass.

4.3. Methods

4.3.1. Ethical Statement

This experiment was conducted under the UK Home Office project licence PP7720523 (Protocol 4). Ethical approval was granted by Newcastle University Animal Welfare and Ethical Review Body (Project ID 549).

4.3.2. Animals and housing

Day old H&N Brown Nick laying hen chicks were delivered to two rearing sites managed by The Lakes Free Range Egg Company. One of these rearing sites was a conventional rearing system with a flat deck design, with a raised area for perching made available to pullets when they were large enough to use the perches. At the other rearing site, birds were reared with dark brooders on a commercial scale. Dark brooders were slowly raised in height as the chicks grew, then were removed completely at 3 weeks of age. This rearing site also had a flat deck design with raised perching areas made available to pullets when they were large enough to access the perches.

At 12 weeks of age, 26 pullets were selected from each rearing site. Average pullets with no signs of keel bone damage (confirmed by palpation) or any other visible health conditions were selected and transported to Newcastle University's Cockle Park Farm, where the experiment was conducted. On arrival at the experimental farm, birds were tagged with leg rings showing an individual ID number and the rearing site that they were collected from. Two rooms were used for temporary housing (one room for each rearing type) for 10 days. These rooms had wood shavings as substrate, minimal enrichment, *ad libitum* food and water, and a light cycle (Table 4.1) to match the conditions of the rearing farms from which they were sourced. The birds were allowed to settle in these rooms with minimal disturbance for two days, and all birds were then subjected to NOTs conducted over a three-day period.

Alternating between the dark brooder and conventional birds, individual birds were placed in an unfamiliar room which was empty except for the novel object, which was an orange plastic cone wrapped in coloured tape. The bird was placed on a mark one metre way from the object, then the experimenter left the room and started a timer. After 10 minutes, the bird was returned to its housing room. Behaviours during the NOT were recorded using a camera mounted on the ceiling of the room and video footage was analysed by a single observer who was blind to the rearing group from which each bird was taken. While other measures (time spent within 10cm, 30cm, and 60cm from the centre of the novel object) were recorded during

the NOT on arrival, very few birds approached the novel object at all, therefore latency to first movement was the only outcome with enough variation between individuals that could be used to assign them to rooms.

Birds were again left to settle for two days with minimal disturbance, then were tested for TI over a period of two days. Alternating between conventional and dark brooder reared birds, each individual was placed on their back in a wooden cradle in an empty room which was adjacent to the housing rooms. The experimenter restrained the bird in the cradle with one hand over the breast and the other hand covering the bird's eyes. When struggling ceased, the hands were removed slowly and a timer was started. When the bird righted itself, the timer was stopped and the duration of TI was recorded. TI was stopped at a maximum of 10 minutes. If the duration of TI was shorter than 10 seconds, the bird was restrained for a second attempt at induction of TI. In all cases, TI was successfully induced within two attempts.

Latency to first movement in the NOT was plotted against duration of TI for each rearing condition (Figure 4.1A). There was no correlation between duration of TI and latency to first movement in the NOT on arrival in conventional (r_{24}=0.274; p = 0.175) or dark brooder birds (r_{24}=-0.044; p = 0.831). The plots were used to assign birds to four housing groups, each with a similar distribution of results from the behavioural tests (Figure 4.1B). Each of these groups contained 13 birds, with 6-7 individuals from each of the rearing conditions. Each of these groups would be housed in a separate room for the remainder of the study.

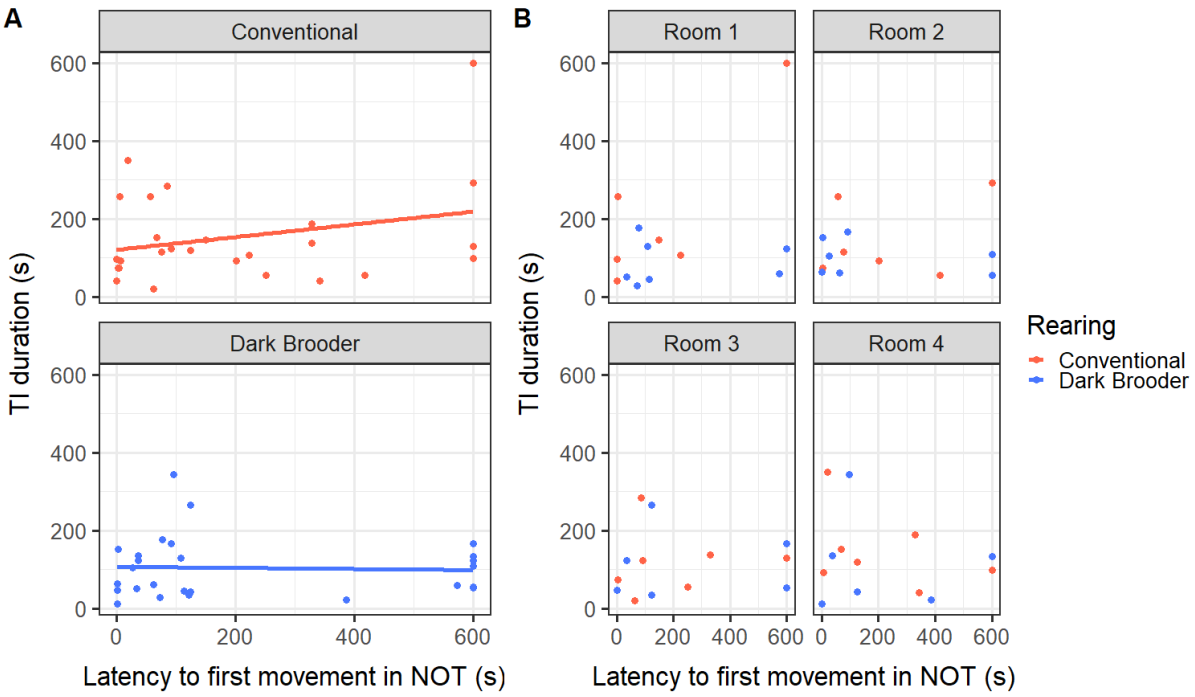

Figure 4.1 - Results of behavioural tests conducted on arrival for assignment of birds to housing rooms. TI duration plotted against latency to first movement in NOT for: ***A)*** *all birds arranged by rearing condition;* ***B)*** *all birds arranged by housing rooms, assigned according to the results of the behavioural tests on arrival. Birds in rooms 1 and 3 were subjected to the UCMS treatment, while birds in rooms 2 and 4 were controls.*

The day after TI was conducted, birds were moved to four identical housing rooms. Each room was divided into two pens. The floor was covered in wood shavings in both pens, which were replaced regularly. The front pen in all rooms was furnished with nesting boxes and perches. The front pen also contained two feeders, one drinker, grit on the floor and minimal enrichment (such as cardboard strips, cardboard tubes, and CDs) which was replenished regularly. The back pen was left barren in all rooms and was used as a space to conduct NOTs and some stress treatments. Birds had access to both pens at all times except during behavioural tests, some stress treatments, and during weekly weighing.

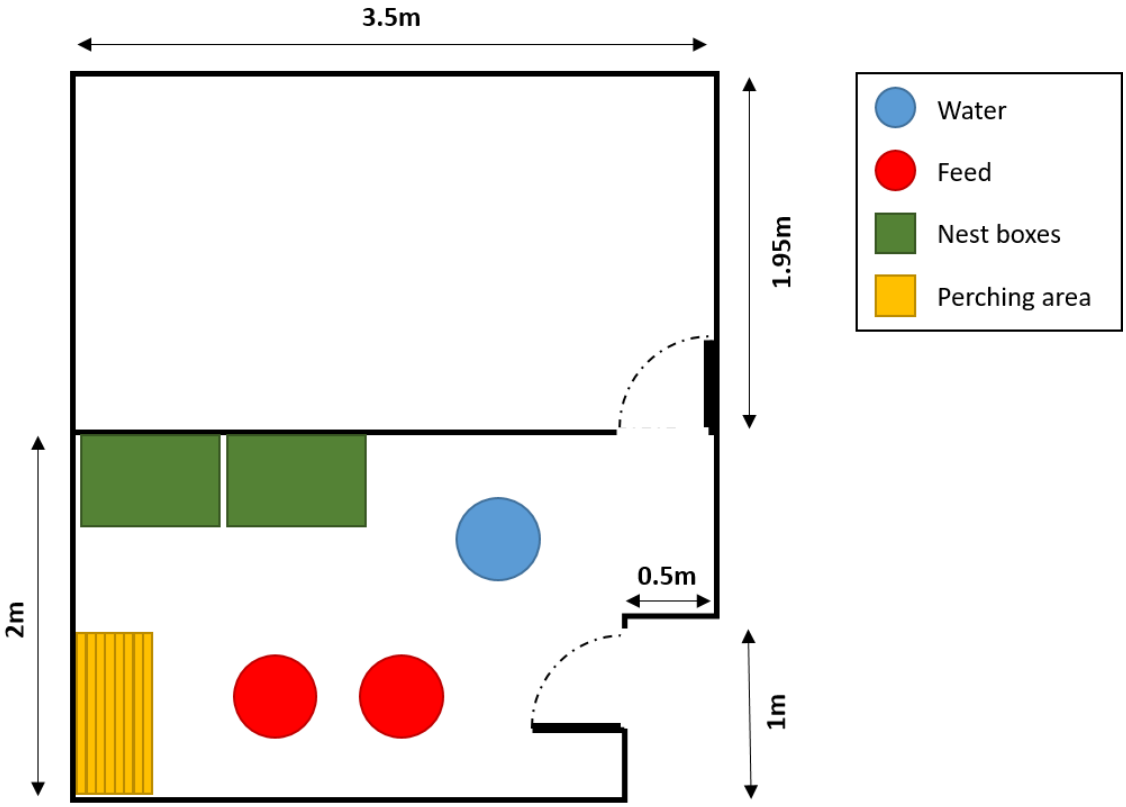

Figure 4.2 - Layout and dimensions of experimental rooms. Back pen was left empty. All four rooms were identical, with 12 birds housed per room.

Birds received ad libitum food and water. Lighting in all rooms was maintained at 20-40 lux, and the hours of lighting (Table 4.1) were increased throughout the study up to a maximum of 15 hours light per day, as would be used on commercial laying farms. The temperature in all rooms was maintained at 21°C, apart from days in which increased temperature was used as a stressor.

Table 4.1 - Hours of lighting for control and UCMS rooms throughout study

Period of study	Weeks of age	Total hours of light per day	Hours for control rooms		Hours for UCMS rooms	
			ON	OFF	ON	OFF
Arrival	12	10	09:00	19:00	09:00	19:00
Pre-treatment	13	10	09:00	19:00	09:00	19:00
	14	10	08:00	18:00	08:00	18:00
	15	10	07:00	17:00	07:00	17:00
	16	11	06:00	17:00	06:00	17:00
UCMS period	17	12	05:00	17:00	Unpredictable	
	18	13	04:00	17:00	Unpredictable	
	19	14	04:00	18:00	Unpredictable	
	20	15	04:00	19:00	Unpredictable	
	21	15	04:00	19:00	Unpredictable	
	22	15	04:00	19:00	Unpredictable	
	23	15	04:00	19:00	Unpredictable	
	24	15	04:00	19:00	Unpredictable	
Dissections	25	15	04:00	19:00	Unpredictable	

4.3.3. Pre-treatment period

During the first week after birds moved into their housing rooms, they were allowed to settle with minimal disturbance. During the second week of the Pre-treatment period, TI was conducted over 2 days. During week 3, one spare bird was removed from each room and was rehomed, leaving 6 from each rearing condition per room. One of the rehomed birds was selected because of aggression from other birds in the room, while the other three rehomed birds were selected randomly, leaving 6 birds from each rearing condition per room. Also during that week, pre-treatment NOTs were conducted over 4 days. In the fourth week of the pre-treatment period, pre-treatment blood samples were collected.

4.3.4. UCMS period

Rooms 1 and 3 were selected for UCMS treatment, while rooms 2 and 4 were controls and were subjected to minimal disturbance. Birds in control rooms were only captured if necessary for weekly weighing, health checks, behavioural tests or blood sampling.

Table 4.2 - Stressors employed during UCMS period in addition to unpredictable lighting

Stressor	Description	Endpoint
Long day	20 hours of light within a 24 hour period.	Lights off after 20 hours (lights on from 04:00 until 00:00)
Short day	4 hours of light within a 24 hour period	Lights off after 4 hours (lights on from 06:00 until 10:00)
Increase temperature	Increase temperature in rooms up to a maximum of 30°C	Temperature reduced to normal after a maximum of 3 hours, or if more than 50% of birds display signs of lethargy, panting, or holding wings open
Remove food and water	Food and water removed from rooms	Food returned to rooms after a maximum of 6 hours. Water returned to rooms after a maximum of 4 hours. Water returned early if more than 50% birds stay in area where water would usually be placed for more than 30 minutes. Food and water both returned to rooms immediately if more than 50% of birds show display signs of lethargy or panting.
Wet bedding	All shavings in rooms soaked with water in the afternoon and left wet overnight	All shavings replaced with dry shavings the following morning
Pack in boxes	All 12 birds in each room packed into one crate	Birds released from crates 3 hours after the first bird was placed in the crate
Isolation	Each bird packed into an individual crate, distributed within the rooms to avoid visual communication between birds	All birds released from crates 1 hour after the first bird was placed in a crate
Chasing	All birds in each room confined to empty back pen, then chased by one person for a maximum of 5 minutes. Repeated up to 4 times per day at unpredictable intervals.	Each bout of chasing was no longer than 5 minutes, with a minimum of 5 minutes between bouts of chasing

Birds in UCMS rooms were subjected to 8 weeks of UCMS treatment beginning at 17 weeks of age, in which they received a mild stressor each day. Mild stressors (Table 4.2) were employed in randomised order to ensure birds could not predict the stressor that would occur each day. On days when behavioural tests or blood sampling was conducted, stressors were applied after testing or sampling.

In addition to these stressors, birds in UCMS rooms were subjected to an unpredictable lighting schedule. They received the same total hours of light within each 24 hour period as the control birds (Table 4.1), however light and darkness were delivered in randomised blocks throughout the day.

Due to birds exhibiting behavioural endpoints, the increased temperature stressor was stopped earlier than 3 hours on all occasions. The minimum duration of time for which birds were exposed to an increased temperature was 1 hour and 5 minutes. The maximum duration of the increased temperature was 1 hour 50 minutes. On one occasion, water was returned early (three hours after removal) due to behavioural endpoints being displayed by more than 50% birds. The food was never returned earlier than planned.

Further behavioural tests and blood samples were performed and collected from all birds during the UCMS period, and eggs were counted starting from onset of lay at 18 weeks and 4 days of age.

4.3.5. Behavioural tests

Pre-treatment TI was tested at 14 weeks of age, during week 2 of pre-treatment period. This was then repeated at 23 weeks of age, during week 6 of UCMS period. Birds were tested in a randomised order, alternating between rooms 1,2,3, and 4 in that order. These TI tests were conducted in an empty pen in each housing room, using the same methods as in the TI tests on arrival. As before, no individuals required more than two attempts to successfully induce TI.

The pre-treatment NOT was conducted at 15 weeks of age, during week 3 of pre-treatment period. All birds were moved to the front pen and the door between the pens was closed. A mat (Figure 4.3C) with circles drawn at 10cm, 30cm, and 60cm from a central point was placed in the back pen, with the centre point directly below a camera. A novel object, here a plastic bottle (base diameter 8cm) containing grit and covered with coloured tape (Figure 4.3A) was placed on the central point of mat. A bird was selected from the front pen and placed on the

starting point of the mat, 1m from the novel object. The observer left the room for 10 minutes, during which the bird was filmed. This was repeated for each bird, with 3 birds tested in each room for 3 days.

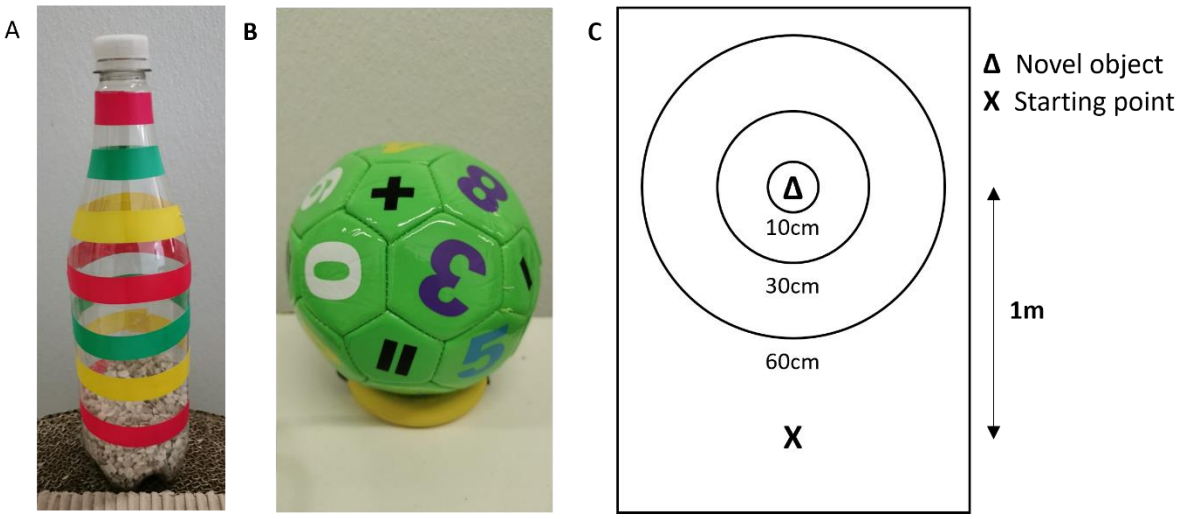

*Figure 4.3 - **A)** Novel object used at pre-treatment; **B)** Novel object used during week 7 of UCMS period; **C)** Diagram of mat used in all NOTs, with circles drawn at 10cm, 30cm, and 60cm from the centre of the novel object, and the point at which the bird was placed 1m from the centre of the novel object*

The NOT was repeated 8 weeks later, during week 7 of UCMS period, using a football (diameter 15cm) with coloured shapes (Figure 4.3B) as the novel object. Behaviours in ethogram in Table 4.3 scored from videos using BORIS by a single observer who was blind to the treatment group and the room from which each bird was taken. Two individuals (one dark brooder UCMS, one conventional control) were excluded from all analysis because of missing video footage. For another individual (conventional UCMS) the recorded time spent freezing was an outlier (6.94 SD above mean). Therefore, this individual was also excluded from analysis of all outcome measures.

Table 4.3 - Ethogram used for scoring behaviours during analysis of video footage from NOTs

Behaviour	Description
First movement	Bird moves away from start point for the first time
60cm	Any part of the bird enters the 60cm circle from another area
30cm	Any part of the bird enters the 30cm circle from another area
10cm	Any part of the bird enters the 10cm circle from another area
Moving	Bird is walking around the arena (at any speed)
Still	The bird is standing in one place, but moving one or more parts of the body
Freezing	The bird is standing in one place, completely still (wings may be lifted)
Door	The bird is standing in a defined area close to the door between the two pens

A second observer scored videos in order to investigate inter-observer reliability in the scoring of each behavioural outcome from of the videos between the two observers. Correlation coefficients (r) ranged from 0.78 to 0.99, suggesting that the measurements taken by the first observer were reliable.

4.3.6. Blood plasma corticosterone

Pre-treatment blood samples were taken at 16 weeks of age, in the final week of the pre-treatment period. Birds were collected individually from their home room and restrained. The skin of the leg was sterilised with 70% ethanol, then a 25G needle was inserted into medial metatarsal vein. Blood was collected from the hub of the needle using up to six heparinised capillary tubes.

Immediately after the first blood sample had been taken, the bird was placed in bag which was hung from a table. After 30 minutes, the bird was released from the bag and a second blood sample was taken from the leg that was not used for the previous sample. If it was not possible to collect blood from the medial metatarsal vein for either sample, blood was collected from the cutaneous ulnar vein in the wing instead. The bird was then returned to the home room. When a bird had been returned to a room, experimenters did not re-enter that room for at least one hour to allow other birds to settle before being selected for blood sampling. Blood samples were collected over a period of 4 days. On each day, three birds were sampled from each room in the order 1,2,3,4, alternating between UCMS and control birds. Blood sampling was repeated after 4 weeks of UCMS (20 weeks of age) and 8 weeks of UCMS

(24 weeks of age). The collected blood was divided between two 1.5ml centrifuge tubes. One tube was stored on ice until an even number of samples had been collected.

CORT concentration in the plasma was quantified using a Corticosterone ELISA kit (ADI-901-097, Enzo Life Sciences, USA) according to the user manual. Assays conducted on 12x 96 well plates. Birds were randomly assigned to each plate, with four birds per plate, one from each treatment group. All 6 samples from each bird were run on the same plate, and all sample were run in triplicate. Each plate also contained 5 standards run in duplicate, with known CORT concentrations 20000, 4000, 800, 160, 32 pg/ml. For some plates, a 6th standard was used with a CORT concentration of 6.4 pg/ml in order to increase detectable concentrations of CORT. Blank, B0 (maximum binding), total activity (TA), and non-specific binding (NSB) control wells were also run in duplicate on each plate.

Plates were read using a Multiskan FC Microplate Photometer (ThermoFisher Scientific, Loughborough, UK) at 405nm, and the optical density of blank wells was subtracted from the optical density of the unknown samples. A 4-parameter logistic curve was plotted with the standards from each plate using mycurvefit.com. The equation of the curve was used to calculate CORT concentration in unknown samples. The concentration of CORT in the plasma was generally very low, and in some cases the concentrations were lower than the 27pg/ml limit recommended by the manufacturers of the ELISA kit, meaning CORT could not be measured accurately in some samples. For samples in which CORT levels were too low to be detectable, the lowest detectable value from that plate was used in analysis.

4.3.7. Hippocampal gene expression

At 25 weeks of age, all birds were killed and dissected over a period of 4 days (3 birds from each room per day. Unpredictable lighting was continued for the remaining UCMS birds throughout the dissections. Individual birds were randomly selected from each room in the order 1,2,3,4.

Birds were sedated with intramuscular ketamine and xylazine, then killed by intravenous injection of sodium pentobarbital (Euthatal). The head was removed immediately after death, and the brain was removed and dissected. One hemisphere of the brain was fresh frozen and stored on dry ice. From the other hemisphere of the brain, the hippocampus was divided into rostral and caudal subregions, which were stored in RNAlater. The samples were moved to a -80°C freezer for long term storage.

During dissections, the mass of the spleen was also recorded to indicate the effects of UCMS on the immune system. When analysing the spleen mass, two individuals were excluded from the analysis because the spleen was damaged during dissection. Another bird was excluded from the analysis because the spleen was abnormally large.

RNA was extracted from the rostral and caudal hippocampus by Chloe Grant during her PhD project. Each tissue sample was added to a ZR BashingBead Lysis tube (Cambridge Biosciences, UK) with 1ml TriSure reagent (Bioline, London, UK). Samples were homogenised in a Qiagen Tissue Lyser II (Qiagen Ltd, Crawley, UK). 200µl chloroform was added to each sample and they were shaken manually for 15 seconds. They were then centrifuged at 13,000 rpm for 15 minutes at 4°C. RNA was extracted from the supernatant using a direct-zol RNA miniprep kit (Zymo Research, Cambridge Biosciences, UK), according to the manufacturer's instructions, and was stored at -80°C.

To produce the cDNA templates which would be used in qPCR for absolute quantification, 1µg RNA from each sample was reverse-transcribed using a Tetro cDNA synthesis kit (Meridian Bioscience, UK). End-point PCR was used to produce DNA standards for each gene of interest: DCX, BDNF, PCNA, and the reference gene LBR. Four reactions were produced for each of these genes. Each 25µl reaction contained 12.5µl MyFi Mix (Bioline, London, UK), 400nM forward primer, 400nM reverse primer (see Table 4.4 for details of gene-specific primers used), 8.5µl sterile distilled water, and 3µl cDNA template which was produced by reverse transcription of RNA from chicken hippocampal tissue.

The BDNF primers were designed and tested by Chloe Grant for her PhD project. The DCX, LBR and PCNA primers were sourced from Armstrong *et al.* (2020b).

Table 4.4 – Gene-specific primers used in PCR

Gene	Accession	Orientation	Primer sequence (5'-3')	Product length (bp)
BDNF	DQ124361.1	Forward	GGCGGACACTTTTGAACACG	74
		Reverse	TGTTTTCCTCACTGGGCTGG	
DCX	NM_204335	Forward	AAGACGGCCCATTCGTTTGA	166
		Reverse	ATTTTTCGGGACCACAGGCA	
LBR	NM_205342	Forward	GGTGTGGGTTCCATTTGTCTACA	80
		Reverse	CTGCAACCGGCCAAGAAA	
PCNA	NM_204170.2	Forward	CAATGCGGATACGTTGGCTC	192
		Reverse	ACAGCATCACCAATGTGGCT	

The reaction tubes were placed in a PCR machine. After a polymerase activation period at 95°C for 60 seconds, the samples were run for 36 cycles, each cycle consisting of denaturation (95°C for 15 seconds), annealing (60°C for 15 seconds) and extension (72°C for 15 seconds) steps. 4µl loading dye was added to each tube, then the solution from each reaction was used for electrophoresis with a 2% agarose gel containing ethidium bromide. Stained bands were excised from the gel, then DNA was extracted from the gel using a MinElute Gel Extraction kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's protocol. The concentration of DNA in each standard was measured using a NanoDrop spectrophotometer (Fisher Scientific, Loughborough, UK). The concentration of DNA in the standards was between 13.3 and 30.6ng/µl.

For each gene, the standard was diluted in five-fold serial dilutions to produce nine standards of known concentration. Six of these standards with the lowest concentration of DNA were used to produce a standard curve for each assay.

Real time quantitative polymerase chain reaction (qPCR) was conducted to quantify expression of DCX, BDNF, PCNA, and LBR genes in the hippocampal samples. Assays were conducted in a 96-well plate layout in a qPCR machine (CFX Connect, Bio-Rad, Watford, UK). Three assays were conducted for each gene of interest, with individual birds randomly assigned to each batch and all treatment groups equally represented within each batch. Each assay included rostral and caudal hippocampal samples with unknown DNA concentration (in duplicate), six standards of known concentration (in duplicate) and two blank controls. Each reaction contained 5µl SensiFAST SYBR No-ROX mix (Bioline, London, UK), 400nM forward primer, 400nm reverse primer (Table 4.4), and 4µl cDNA (from samples of unknown concentration), DNA standard (for reactions in the standard curve), or sterile distilled water (for blank controls). All samples and standards underwent 40 cycles of a three-step thermal cycling protocol (denaturation at 95°C for 15 seconds, annealing at 60°C for 10 seconds, extension 72°C for 15 seconds). The R^2 of the standard curves ranged between 0.987 and 1.000, and the efficiency of the primers ranged between 92.6% and 115.8%. Single melting peaks were obtained for all primer pairs.

Maestro-CFX software (Bio-Rad, California, USA) was used to calculate molar values of DNA in the unknown samples according to the standard curve. Within each individual, molar values for DCX, BDNF, and PCNA were divided by the molar value for the reference gene LBR. These values were $\log_{10}$ transformed before statistical analysis. Individuals with ratios more than 3

SD from the mean were excluded from the analysis. This included three individuals for DCX, three individuals for BDNF, and two individuals for PCNA.

4.3.8. Statistical analysis

All statistical analysis was conducted in R Studio (R Core Team, 2023). For all linear mixed models analysis was conducted using the 'afex' package (Singmann *et al.*, 2023), with post-hoc pairwise comparisons conducted using the emmeans package (Lenth, 2024).

TI data was analysed using mixed effects Cox regression using the 'survival' (Thernau, 2023) and 'coxme' (Thernau, 2022) packages in R, with time in TI as the dependent variable. The between-subjects independent variables were stress and rearing. Time-point (before or after UCMS period) was be included as a within-subject repeated measure.

The data from the NOTs were analysed using linear mixed models. The dependent variables, such as latency to first movement, time spent moving, time spent freezing, time spent still, and time spent near the door, were analysed using three-way ANOVAs. Stress and rearing were included as between-subjects factors, while time-point (before and after the UCMS period) was included as a within-subject repeated measure. Time spent in the 30cm, and 60cm circles during the NOTs was corrected by dividing the time spent in each circle by the area of that circle. When calculating the area of the circles, The 10cm circle was excluded from the area of the 30cm circle, and the 30cm circle was excluded from the area of the 60cm circle. Time spent in the 10cm circle was excluded from analysis because the novel objects covered a large area of the inner circle, and the area covered by each object was different, making time spent in the 10cm circle difficult to interpret.

A Four-way ANOVA was used to analyse the time spent in these circles, with stress and rearing included as between-subjects factors, and both time-point (before and after the UCMS period) and "time in circles" (with two levels: 30cm and 60cm) included as within-subject repeated measures. All interactions among these factors were incorporated into the model. A separate three-way mixed ANOVA was used to analyse the total time spent in the 30cm and 60cm circles combined, with stress, rearing included as between-subject factors, and time-point included as a within-subject factor.

The CORT concentrations of the samples were Z-scored within plates. Unexpectedly, there were differences in CORT between UCMS and control birds even at pre-treatment. To account for this, the Z-scores were adjusted to make the baseline CORT at pre-treatment 0. Three birds

from room 2 were excluded from the analysis because abnormally high values from birds in that room were recorded from all samples taken on one particular day. The data from blood plasma corticosterone was analysed using a four-way mixed ANOVA, with stress and rearing as between-subjects factors. Time-point (pre-treatment, UCMS week 4, or UCMS week 8) and restraint (before or after restraint in bag) were included as within-subject repeated measures. All interactions between these factors were included in the models. Differences between the housing rooms were explored using a separate four-way mixed ANOVA.

Different patterns were detected in the body mass data between 13-18 weeks of age (before onset of lay) and 19-25 weeks of age (after onset of lay). Therefore, body mass was analysed for these two time periods separately, using linear mixed models, with age as continuous variables and stress and rearing as between subject factors. The effects of stress and rearing on body mass at death was analysed using two-way ANOVA, and spleen mass was analysed with an ANCOVA, including body mass at death as a covariate.

Effects of stress and housing rooms on eggs laid within the first 10 days after onset of lay were analysed using chi-squared tests. Also, a sigmoid curve was fitted for egg production in each room with the equation $\frac{a}{1+\exp\left(\frac{m-time}{s}\right)}$, using the nls function in R as in Montalcini, Petelle and Toscano (2023). Here, *time* was the number of days from the date that the first egg was laid, *a* was the level at which the number of eggs laid stabilised, *m* was the time at *a*/2 as an indication of the inflection point, and s was the steepness of the curve at *a*/2. The sigmoid curves were plotted using egg data from the day that the first egg was laid until the end of UCMS period (45 days), and parameter estimates *a* and *m* were reported to provide descriptive information about egg production in each room.

The data from hippocampal gene expression was analysed using three-way mixed ANOVAs, with stress and rearing as between-subject factors, and subregion of the hippocampus as a within-subject repeated measure. All interactions between these factors were included in the model. This analysis was carried out separately for each of the three dependent variables: the $\log_{10}$ ratios for expression of DCX, BDNF, and PCNA relative to the reference gene LBR. Ratios for DCX and PCNA were transformed by adding a constant of 1 to all values after $\log_{10}$ transformation.

4.4. Results

4.4.1. Tonic immobility

There was no main effect of stress ($\chi^2_1 = 0.04$, $p = 0.849$) or rearing ($\chi^2_1 = 1.52$, $p = 0.218$) on the time taken for birds to self-right after successful induction of TI. There was a main effect of time-point ($\chi^2_1 = 4.17$, $p = 0.041$), with longer TI durations at week 6 than at pre-treatment overall (Figure 4.4). There was also an interaction between time-point and stress ($\chi^2_1 = 4.93$, $p = 0.026$).

Post hoc tests show there was a significantly longer time to self-righting in UCMS birds at week 6 compared to pre-treatment ($p = 0.001$), but no difference in TI duration between the two time-points in control birds ($p = 0.926$). There was no significant difference between UCMS and controls birds at pre-treatment ($p = 0.084$), or at week 6 ($p = 0.143$).

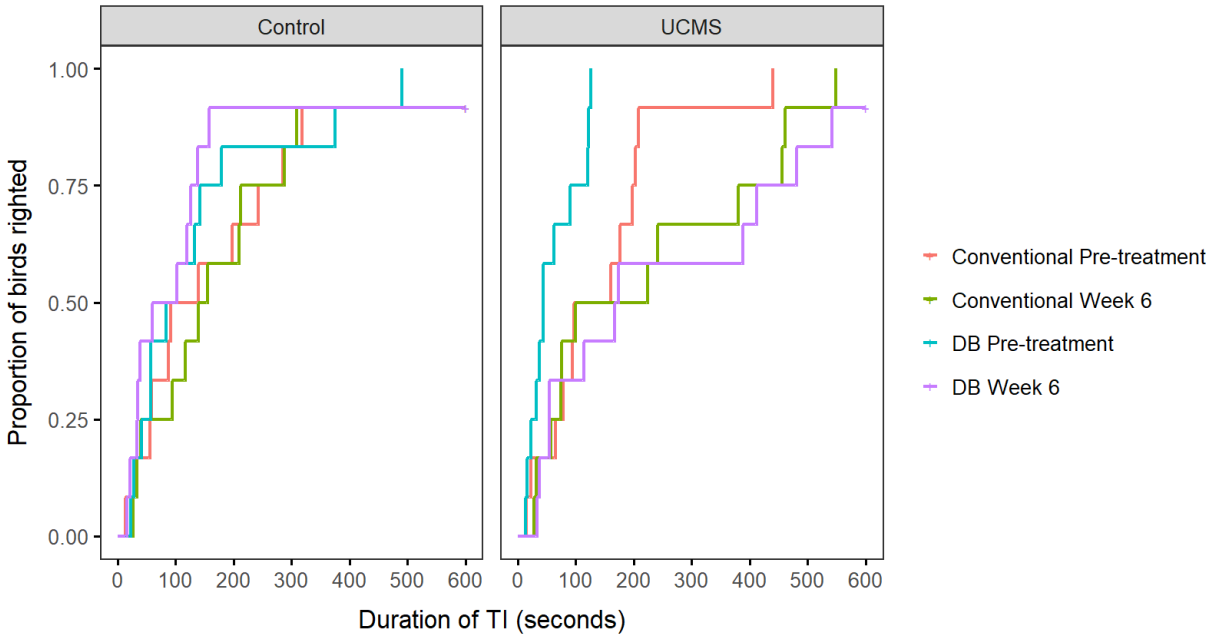

Figure 4.4 – Survival distribution of the time taken (s) for UCMS and control birds to right during TI tests during the pre-treatment period and during week 6 of the UCMS period.

There was no interaction between rearing and time-point ($\chi^2_1 = 1.28$, $p = 0.257$), and no three-way interaction between rearing, time-point, and stress ($\chi^2_1 = 2.66$, $p = 0.103$) on TI duration.

4.4.2. Latency to first movement during novel object tests

There was significantly greater latency to first movement at week 7 than during the pre-treatment NOT ($F_{1,41} = 7.41$, $p = 0.009$) across all treatments (Figure 4.5). There was no main effect of stress ($F_{1,41} = 1.44$, $p = 0.237$) or rearing ($F_{1,41} = 0.04$, $p = 0.837$) on latency to first movement.

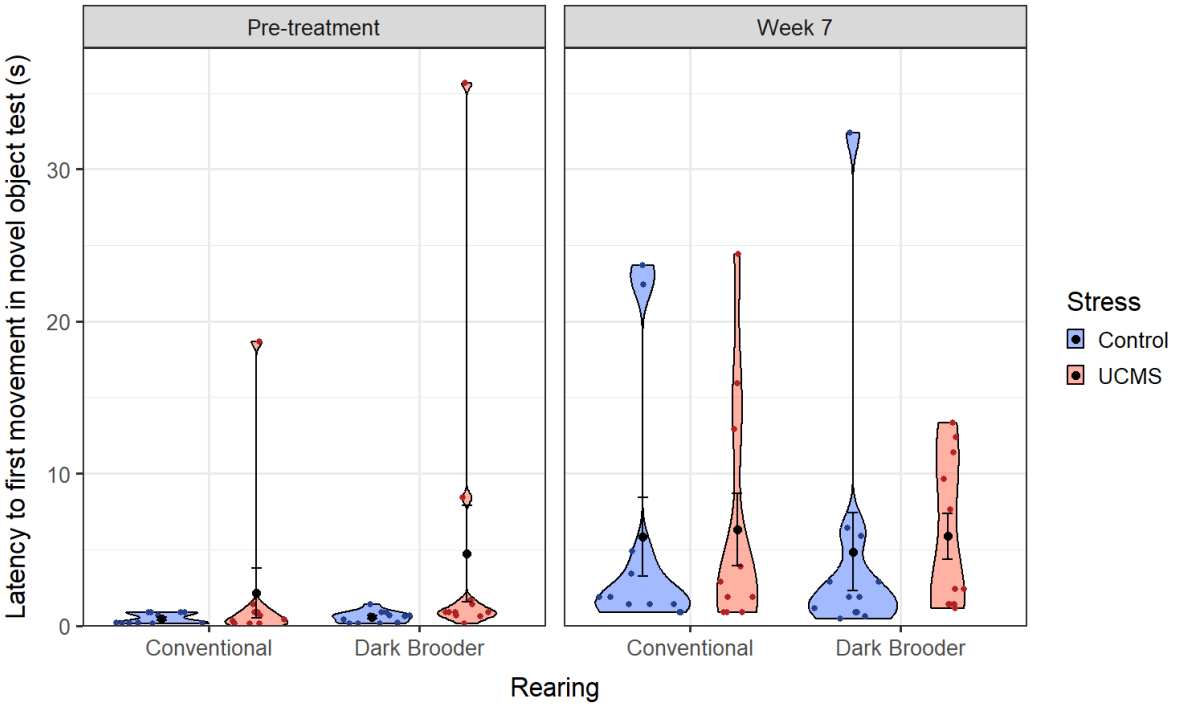

Figure 4.5 – Distributions of time (s) until first movement in NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE latency to first movement.

There were no significant interactions between stress and rearing ($F_{1,41}$ = 0.25, p = 0.622), stress and time-point ($F_{1,41}$ = 0.63, p = 0.433), or rearing and time-point ($F_{1,41}$ = 0.55, p = 0.461). There was no significant three-way interaction between stress, rearing and time-point ($F_{1,41}$ = 0.13, p = 0.722).

4.4.3. Time spent moving, still and freezing during novel object tests

Overall, the birds spent significantly more time moving during the NOT at week 7 than during the pre-treatment test ($F_{1,41}$ = 15.08, p < 0.001) (Figure 4.6). There was also a significant interaction between time-point and stress ($F_{1,41}$ = 4.15, p = 0.048). When averaged across the levels of rearing, post hoc pairwise comparisons show that UCMS birds spent significantly more time moving at week 7 than at pre-treatment (p < 0.001), however there was no difference in time spent moving between the two time-points in control birds (p = 0.194). There was no overall effect of stress on time spent moving ($F_{1,41}$ = 0.27, p = 0.605), with no difference between UCMS and control birds either at pre-treatment (p = 0.321) or week 7 (p = 0.081).

There was no main effect of rearing ($F_{1,41}$ = 0.13, p = 0.720), and no significant interactions between rearing and stress ($F_{1,41}$ = 0.40, p = 0.531) or rearing and time-point ($F_{1,41}$ = 2.39, p =

0.130). There was no significant three-way interaction between the effects of stress, rearing and time-point ($F_{1,41}$ = 0.01, p = 0.906).

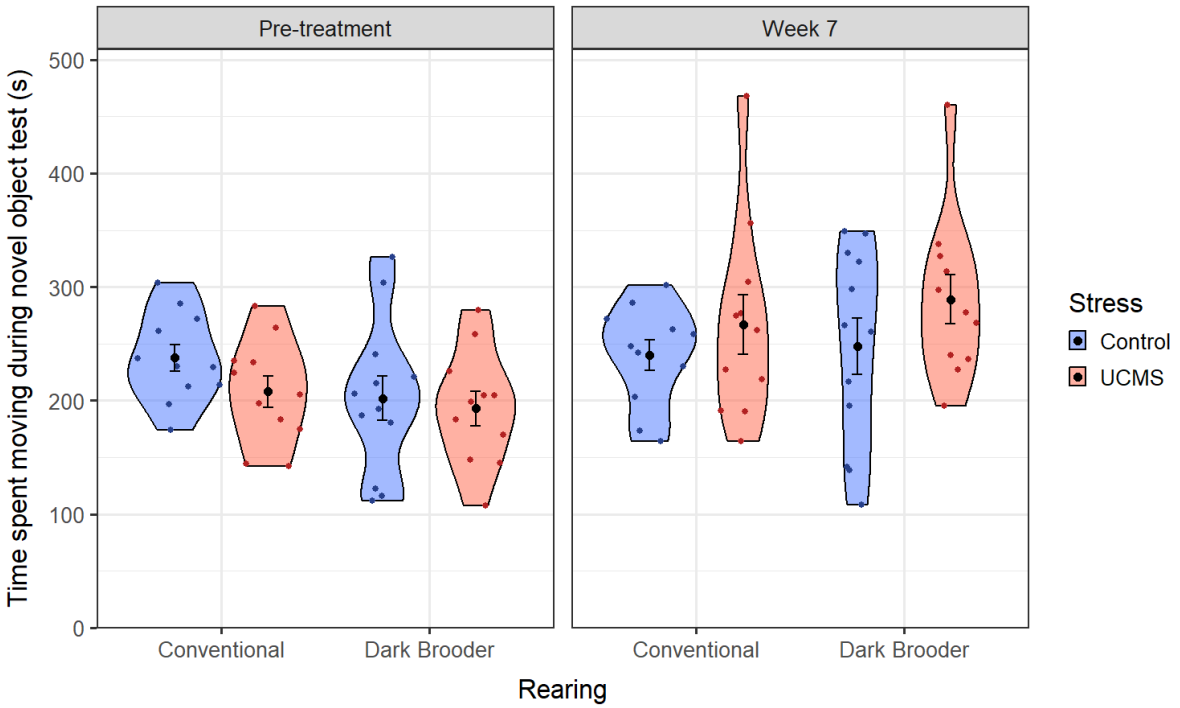

Figure 4.6 – Distributions of time spent moving (s) in NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE time spent moving.

Birds spent significantly less time still during the NOT at week 7 than during pre-treatment ($F_{1,41}$ = 10.54, p = 0.002). There was no main effect of stress ($F_{1,41}$ = 0.53, p = 0.470) or rearing ($F_{1,41}$ = 0.14, p = 0.714) on time spent still.

There were no significant interactions between stress and rearing ($F_{1,41}$ = 0.25, p = 0.622), stress and time-point ($F_{1,41}$ = 2.27, p = 0.140), or rearing and time-point ($F_{1,41}$ = 1.88, p = 0.178) on time spent still (Figure 4.7). There was also no three-way interaction between stress, rearing, and time-point ($F_{1,41}$ = 0.02, p = 0.878).

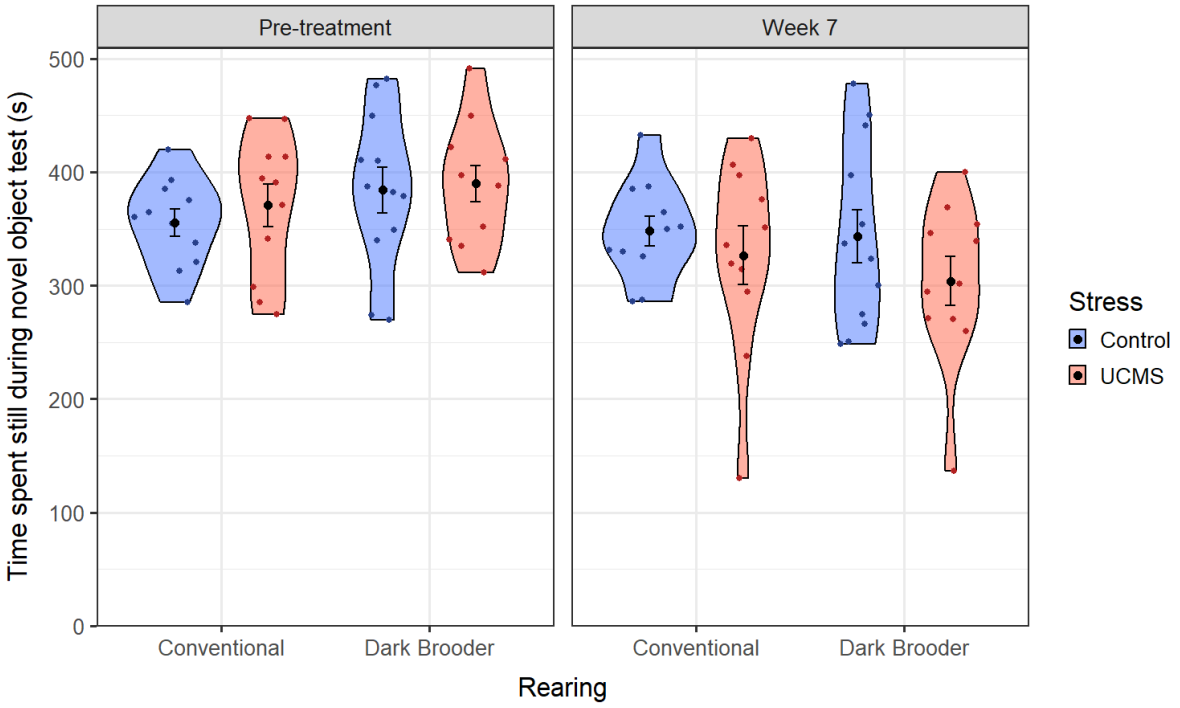

Figure 4.7 – Distributions of time spent still (s) in NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE time spent still.

There was no main effect of stress ($F_{1,41} = 0.23$, $p = 0.634$) or rearing ($F_{1,41} = 0.22$, $p = 0.638$) on time spent freezing (Figure 4.8). Birds tend to spend less time freezing at week 7 than at pre-treatment, however the difference between the two time-points did not reach the threshold of significance ($F_{1,41} = 4.06$, $p = 0.051$).

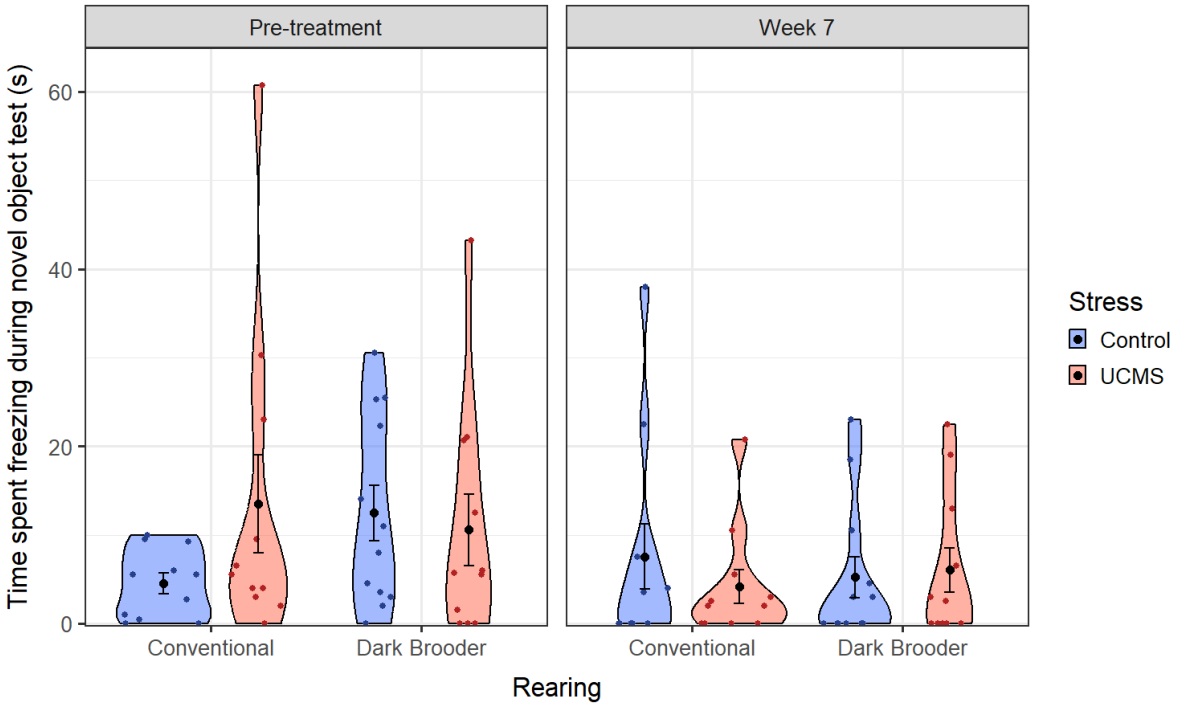

Figure 4.8 – Distributions of time spent freezing (s) in NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE time spent freezing.

There were no significant interactions between stress and rearing ($F_{1,41}$ = 1.14, p = 0.293), stress and time-point ($F_{1,41}$ = 0.50, p = 0.484), or rearing and time-point ($F_{1,41}$ = 0.37, p = 0.545) on time spent freezing. There was also no three-way interaction between stress, rearing, and time-point ($F_{1,41}$ = 2.82, p = 0.101).

4.4.4. Time spent near door during novel object tests

During the NOTs at week 7, the birds spent significantly more time close to the door between the pens than at pre-treatment ($F_{1,41}$ = 6.22, p = 0.017) (Figure 4.9). Dark brooder reared birds spent significantly more time close to the door than conventionally reared birds ($F_{1,41}$ = 5.64, p = 0.022).

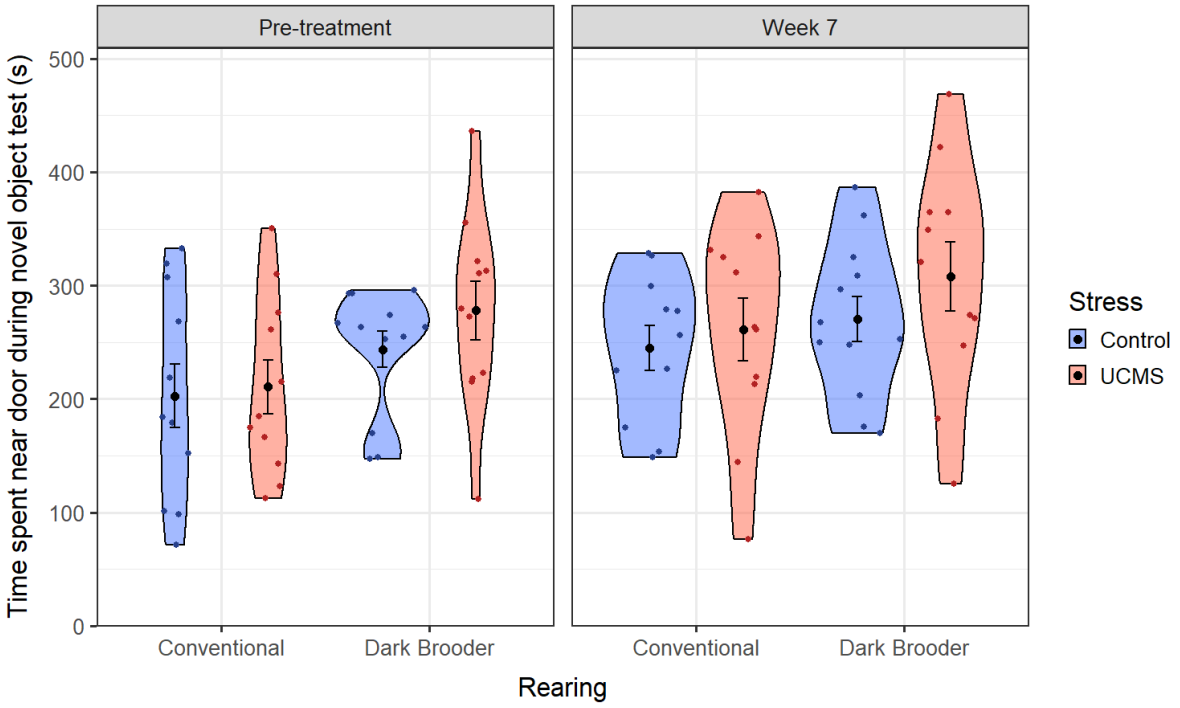

Figure 4.9 – Distributions of time spent in the area near the door (s) during NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE time spent near the door.

There was no significant main effect of stress ($F_{1,41}$ = 1.61, p = 0.212), and no interactions between stress and rearing ($F_{1,41}$ = 0.40, p = 0.531) or stress and time-point ($F_{1,41}$ = 0.04, p = 0.848). There was also no interaction between rearing and time-point ($F_{1,41}$ = 0.36, p = 0.554) and no three-way interaction between stress, rearing, and time-point ($F_{1,41}$ = 0.01, p = 0.938).

4.4.5. Time spent close to the novel object

After correcting for the area of the circles, there was a significant main effect of circle ($F_{1,123}$ = 10.92, p = 0.001) with more time spent in the 60cm circle than the 30cm circle across the two time-points (Figure 4.10). There was also a main effect of time-point ($F_{1,123}$ = 44.96, p < 0.001) with birds spending significantly less time in circles at week 7 than at pre-treatment.

There was no main effect of stress ($F_{1,41}$ = 0.17, p = 0.685), and no main effect of rearing ($F_{1,41}$ < 0.01, p = 0.976), however there was a significant interaction between rearing and circle ($F_{1,123}$ = 4.33, p = 0.039). Post hoc pairwise comparisons show that there was less time spent in the 30cm circle than the 60cm by conventional birds (p < 0.001), but no difference in time spent between the two circles by dark brooder birds (p = 0.383). There was no overall difference between dark brooder and conventional birds for time spent in the 30cm (p = 0.230) or 60cm circles (p = 0.212).

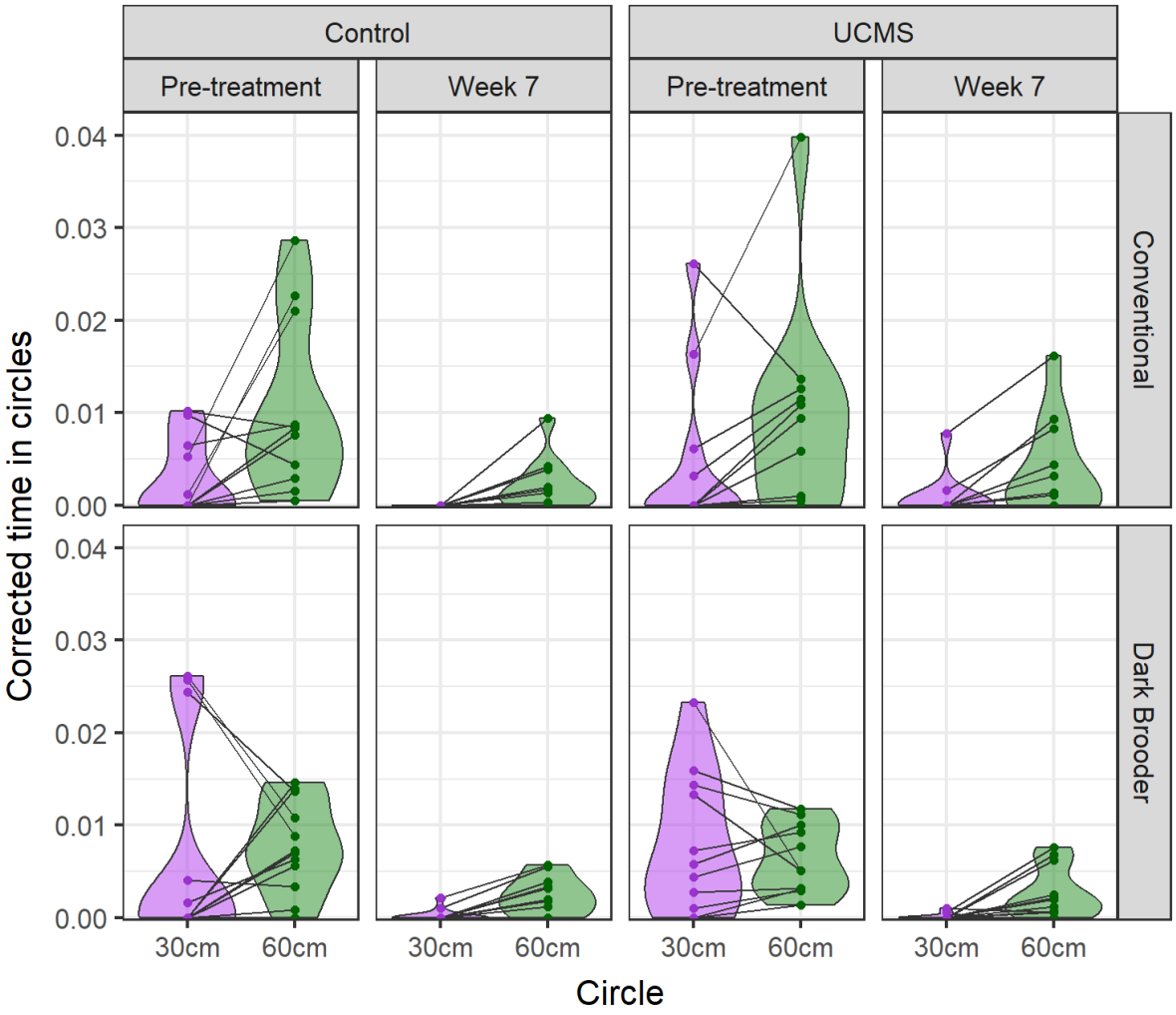

Figure 4.10 - Distributions of time spent in 30cm and 60cm circles during NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Time in circles was corrected by dividing the time spent in each circle(s) by the area of the circles (cm^2). The 10cm circle was excluded from the area of the 30cm circle, and the 30cm circle was excluded from the area of the 60cm circle. Black points and error bars represent mean ±1SE corrected time spent in each circle.

There were no significant interactions between stress and rearing ($F_{1,41}$ = 0.13, p = 0.717), stress and time-point ($F_{1,123}$ = 0.10, p = 0.752), or stress and circle ($F_{1,41}$ = 0.30, p = 0.585), and no significant interactions between time-point and rearing ($F_{1,123}$ = 0.18, p = 0.673) or time-point and circle ($F_{1,123}$ = 0.05, p = 0.820).

There were no significant three-way interactions of stress*rearing*circle ($F_{1,123}$ < 0.01, p = 0.965), stress*rearing*time-point ($F_{1,123}$ = 0.05, p = 0.827), stress*circle*time-point ($F_{1,123}$ = 0.95, p = 0.331), or rearing*circle*time-point ($F_{1,123}$ = 3.56, p = 0.061). There was no four-way interaction between stress, rearing, circle and time-point ($F_{1,123}$ < 0.01, p = 0.939).

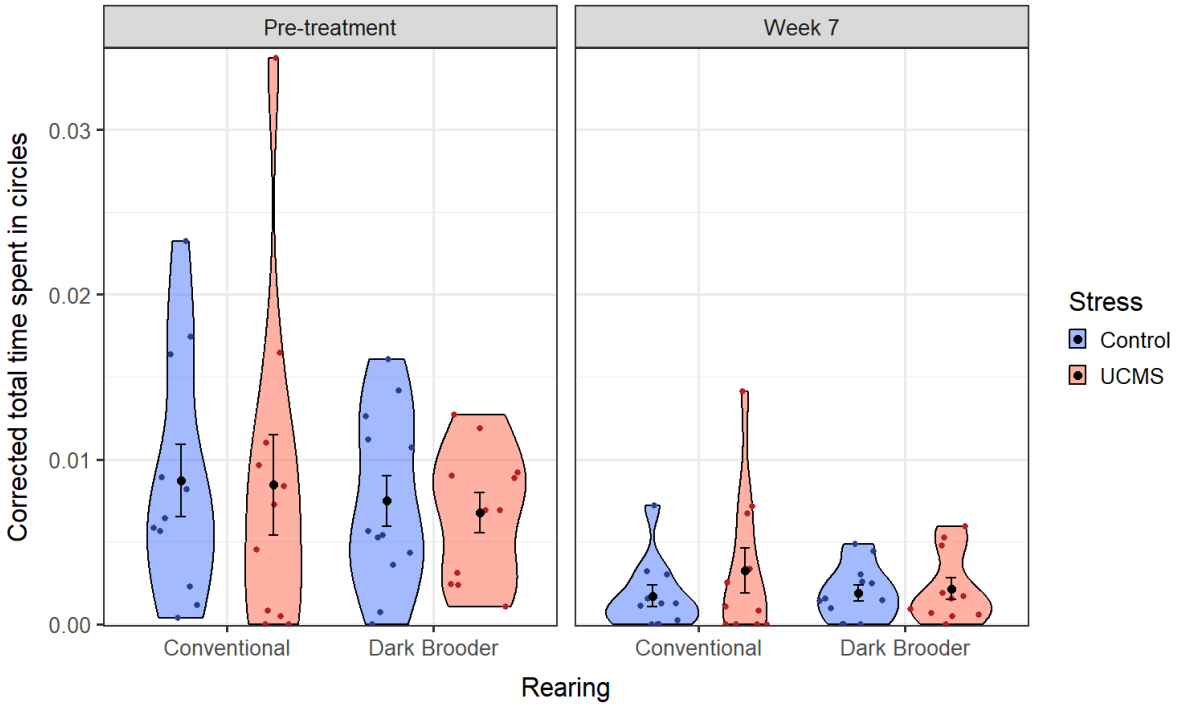

Figure 4.11 - Distributions of total time spent inside circles during NOTs during the pre-treatment period and during week 7 of the UCMS period, for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE time spent near the door.

After correcting for the combined area of the 30cm and 60cm circles, there was significantly less total time spent in circles at week 7 than at pre-treatment ($F_{1,41} = 27.55$, $p < 0.001$) (Figure 4.11). There was no main effect of UCMS ($F_{1,41} = 0.03$, $p = 0.863$) or rearing ($F_{1,41} = 0.65$, $p = 0.424$) on total time spent in circles. There were no two-way interactions between stress and rearing ($F_{1,41} = 0.13$, $p = 0.720$), stress and time-point ($F_{1,41} = 0.42$, $p = 0.521$), or rearing and time-point ($F_{1,41} = 0.22$, $p = 0.643$), and no three-way interaction between the effects of stress, rearing, and time-point on total time spent in circles ($F_{1,41} = 0.04$, $p = 0.844$).

4.4.6. Blood plasma corticosterone

Across all stress and rearing groups, CORT levels (Figure 4.12) differed significantly between the time-points at which blood samples were taken throughout the study ($F_{2,205} = 4.52$, $p = 0.012$). Post hoc pairwise comparisons show significantly lower CORT in week 4 than the baseline CORT at week 0 ($p = 0.011$), but no difference in CORT levels between week 0 and week 8 ($p = 0.101$) or between week 4 and week 8 ($p = 0.660$).

The main effect of chronic stress did not reach threshold for significance ($F_{1,41} = 3.68$, $p = 0.062$), though CORT tended to be slightly higher in UCMS birds than controls. There was no main effect of rearing on blood plasma CORT ($F_{1,41} = 1.86$, $p = 0.180$). Measured CORT levels

increased slightly after restraint by hanging in a bag (acute stressor), but this increase was not statistically significant ($F_{1,205} = 3.09$, $p = 0.080$).

There was a three-way interaction of stress, rearing and restraint ($F_{1,205} = 4.51$, $p = 0.035$) on blood plasma CORT levels. Post hoc pairwise comparisons were used to investigate the nature of this interaction from three different angles. There was no significant effect of UCMS on baseline CORT (before restraint) in conventional ($p = 0.184$) or dark brooder ($p = 0.490$) birds. However, after restraint, there was significantly higher CORT in UCMS than controls in dark brooder birds only ($p = 0.018$). There was no effect of UCMS on CORT in conventional birds after restraint ($p = 0.681$).

In control birds, there was no effect of rearing on CORT before restraint ($p = 0.945$), but there was higher CORT in conventional than dark brooder birds after restraint ($p = 0.028$). In UCMS birds, there was no significant effect of rearing on CORT either before ($p = 0.442$) or after ($p = 0.733$) restraint.

In control conventional birds only, there is significantly higher CORT after restraint than at baseline ($p = 0.027$). CORT did not increase significantly after restraint in control dark brooder birds ($p = 0.227$), or in UCMS birds reared with ($p = 0.117$) or without a dark brooder ($p = 0.376$).

There were no significant two-way interactions between stress and rearing ($F_{1,41} = 0.27$, $p = 0.609$), restraint ($F_{1,205} = 0.38$, $p = 0.538$), or week ($F_{2,205} = 1.07$, $p = 0.344$). Also, there were no two-way interactions between rearing and restraint ($F_{1,205} = 2.13$, $p = 0.146$), rearing and week ($F_{2,205} = 1.05$, $p = 0.350$), or restraint and week ($F_{2,205} = 0.53$, $p = 0.588$).

There were no significant three-way interactions of stress*rearing*week ($F_{2,205} = 2.01$, $p = 0.136$), stress*restraint*week ($F_{2,205} = 0.62$, $p = 0.539$), or rearing*restraint*week ($F_{2,205} = 0.27$, $p = 0.761$). There was no significant four-way interaction between stress, rearing, restraint, and week.

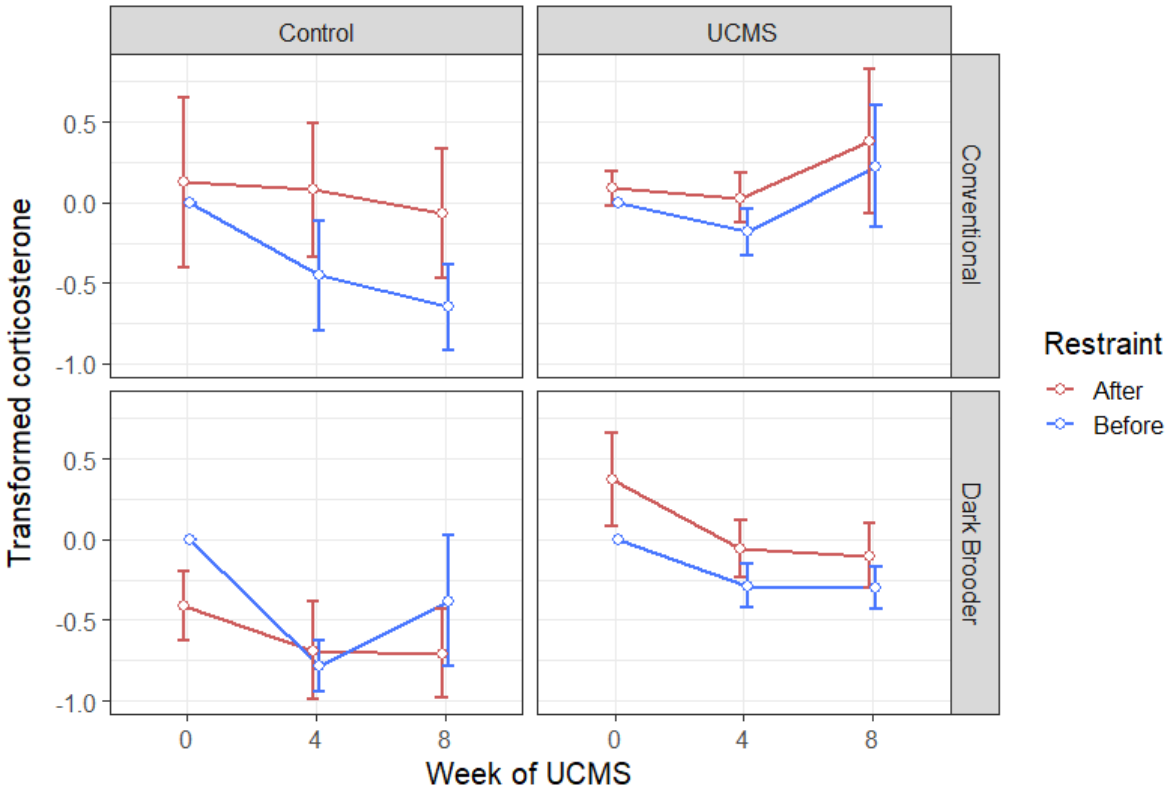

Figure 4.12 - Effects of chronic stress and rearing on change from baseline (pre-treatment) blood plasma corticosterone concentration before and after acute stressor (restraint in a bag) at week 0 (pre-treatment), week 4, and week 8 of the UCMS period.

A separate four-way ANOVA was conducted to investigate differences between rooms. There was no difference between the four rooms ($F_{3,37}$ = 1.24, p = 0.309). As before, there was a significant main effect of week ($F_{2,185}$ = 3.83, p = 0.023), but no main effects of rearing ($F_{1,37}$ = 2.54, p = 0.120) or restraint ($F_{1,185}$ = 2.81, p = 0.096). There were no significant interactions between room and week ($F_{6,185}$ = 0.95, p = 0.460), rearing ($F_{3,37}$ = 1.59, p = 0.208), or restraint ($F_{3,185}$ = 0.18, p = 0.908), and no four-way interaction between room, week, rearing and restraint week ($F_{6,185}$ = 0.53, p = 0.783).

4.4.7. Body and spleen mass

Different patterns were observed before and after onset of lay at 18 weeks and 4 days of age (Figure 4.13). Therefore, these were analysed separately. Between 13-18 weeks of age, body mass was predicted by age ($F_{1,274}$ = 138.38, p < 0.001). There was no effect of stress ($F_{1,274}$ = 0.28, p = 0.594) or rearing ($F_{1,274}$ = 0.09, p = 0.770) on body mass at 13-18 weeks of age, and no two-way interactions between stress and rearing ($F_{1,274}$ = 0.96, p = 0.328), stress and age ($F_{1,274}$ = 0.11, p = 0.736), or rearing and age ($F_{1,274}$ = 0.04, p = 0.845). There was also no three-way interaction between stress, rearing, and age ($F_{1,274}$ = 0.47, p = 0.492).

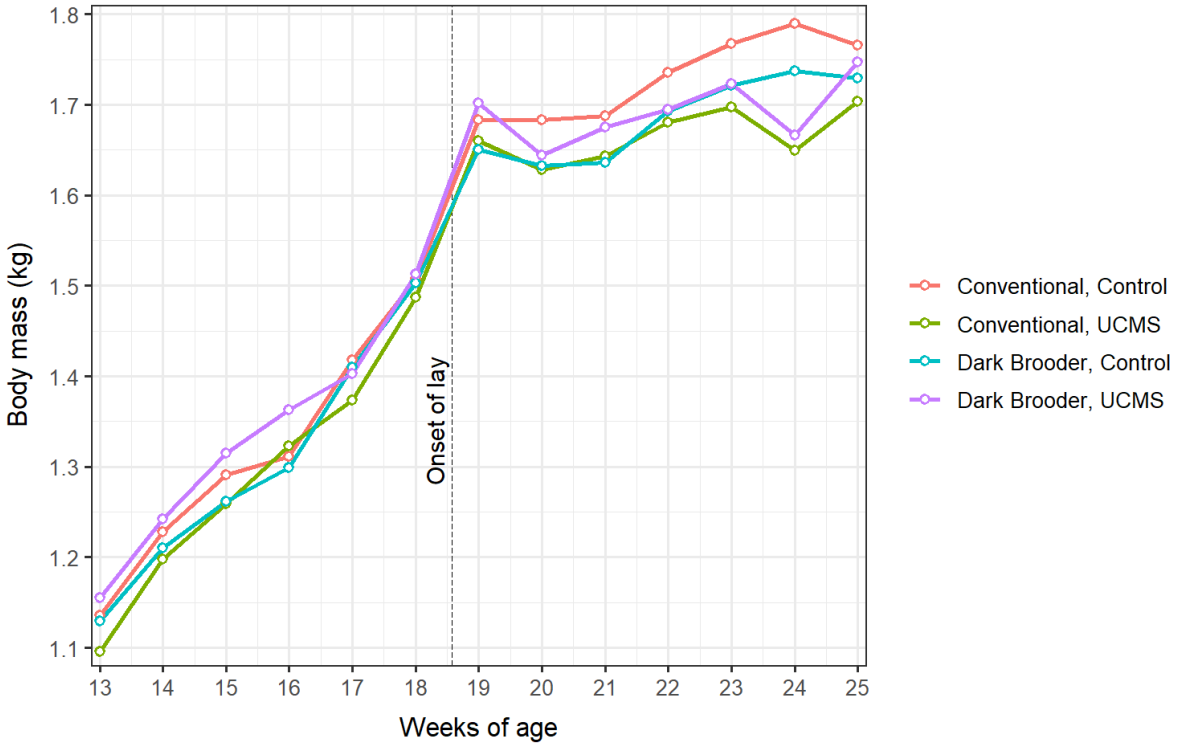

Figure 4.13 - Mean weekly body mass (kg) of laying hens between 13 and 25 weeks of age

Though the pattern was different between 19-25 weeks of age, body mass was also predicted by age here ($F_{1,321}$ = 8.33, p = 0.004). Again, there was no effect of stress ($F_{1,321}$ = 0.75, p = 0.388) or rearing ($F_{1,321}$ = 0.03, p = 0.856) on body mass. There were no two-way interactions between stress and rearing ($F_{1,321}$ = 0.05, p = 0.827), stress and age ($F_{1,321}$ = 1.39, p = 0.240), or rearing and age ($F_{1,321}$ < 0.01, p = 0.976), and no three-way interaction between stress, rearing, and age ($F_{1,321}$ < 0.01, p = 0.982).

Final body mass at dissection (25 weeks of age) was also analysed separately. There was no main effect of stress ($F_{1,44}$ = 1.16, p = 0.288) or rearing ($F_{1,44}$ = 0.55, p = 0.464) on final body mass (Figure 4.14), and no interaction between stress and rearing on final body mass ($F_{1,44}$ = 1.11, p = 0.298).

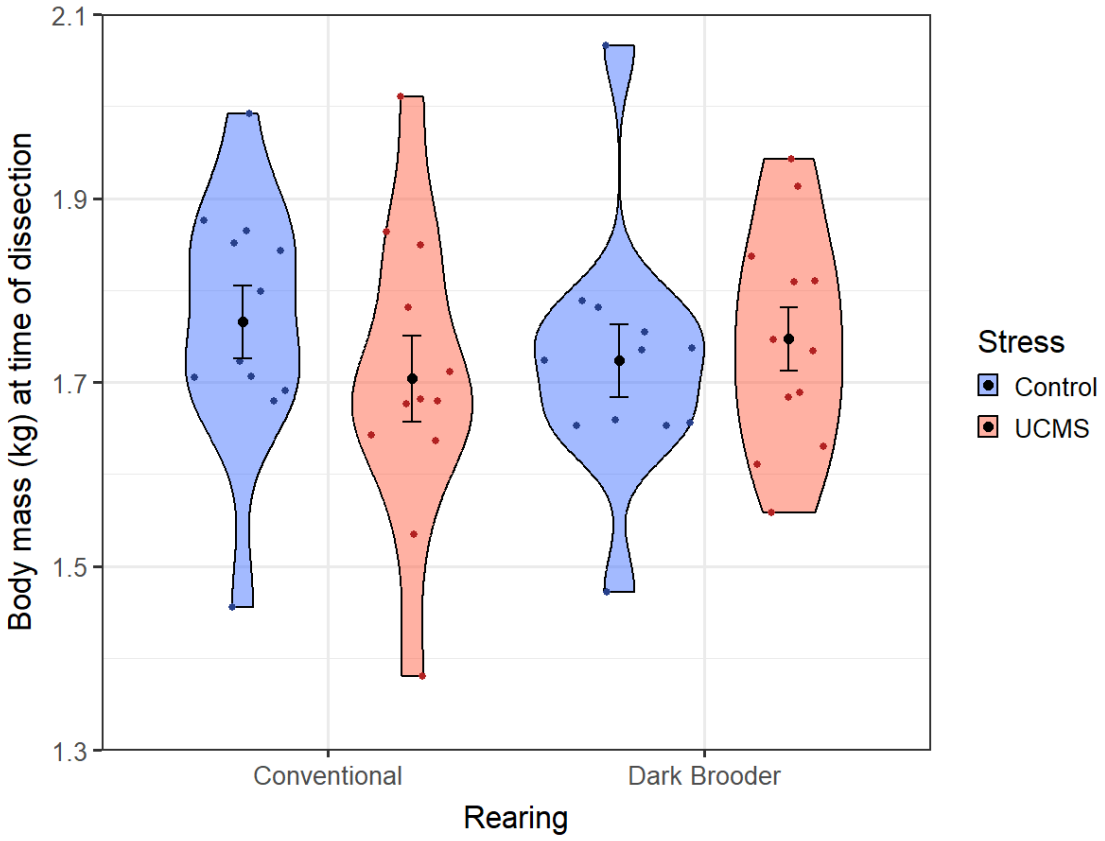

Figure 4.14 – Distributions of body mass (kg) at 25 weeks of age for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE body mass.

Body mass covaried with spleen mass ($F_{1,40}$ = 9.09, p = 0.004) at 25 weeks of age. After correction for body mass, there was no effect of stress ($F_{1,40}$ = 1.10, p = 0.301) or rearing ($F_{1,40}$ = 2.03, p = 0.162), and no interaction between the effects of stress and rearing on spleen mass ($F_{1,40}$ = 0.10, p = 0.750) Figure 4.15).

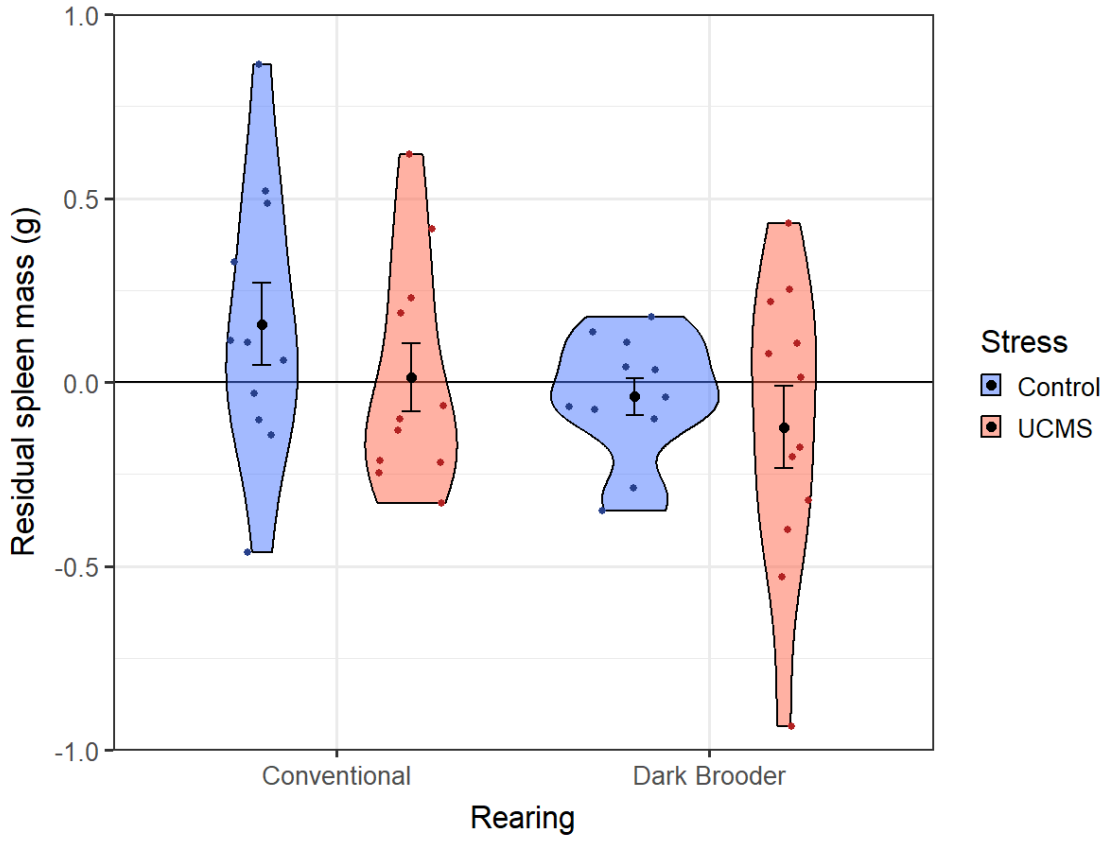

Figure 4.15 – Distributions of residual spleen mass (after correction for body mass at 25 weeks of age) for control and UCMS birds reared conventionally or with a dark brooder. Black points and error bars represent mean ±1SE residual spleen mass.

4.4.8. Egg production

In the first 10 days from the onset of lay, the total number of eggs laid by UCMS birds did not differ from the total number of eggs laid by control birds ($\chi^2_1 = 0.83$, $p = 0.361$). Also, eggs laid in the first 10 days did not differ by room ($\chi^2_3 = 3.67$, $p = 0.299$).

Figure 4.16 shows sigmoid curves fitted to the number of eggs laid in each room per day, from which parameter estimates were calculated. The number of days until the inflection point of the curves were very similar for the control rooms, 2 and 4, with *m* estimates of 9.01 and 9.11 days respectively. The inflection point for room 3 (UCMS) was slightly later than in the control rooms at 9.99 days, while the inflection point for room 1 (UCMS) was slightly earlier than the control rooms at 7.25 days.

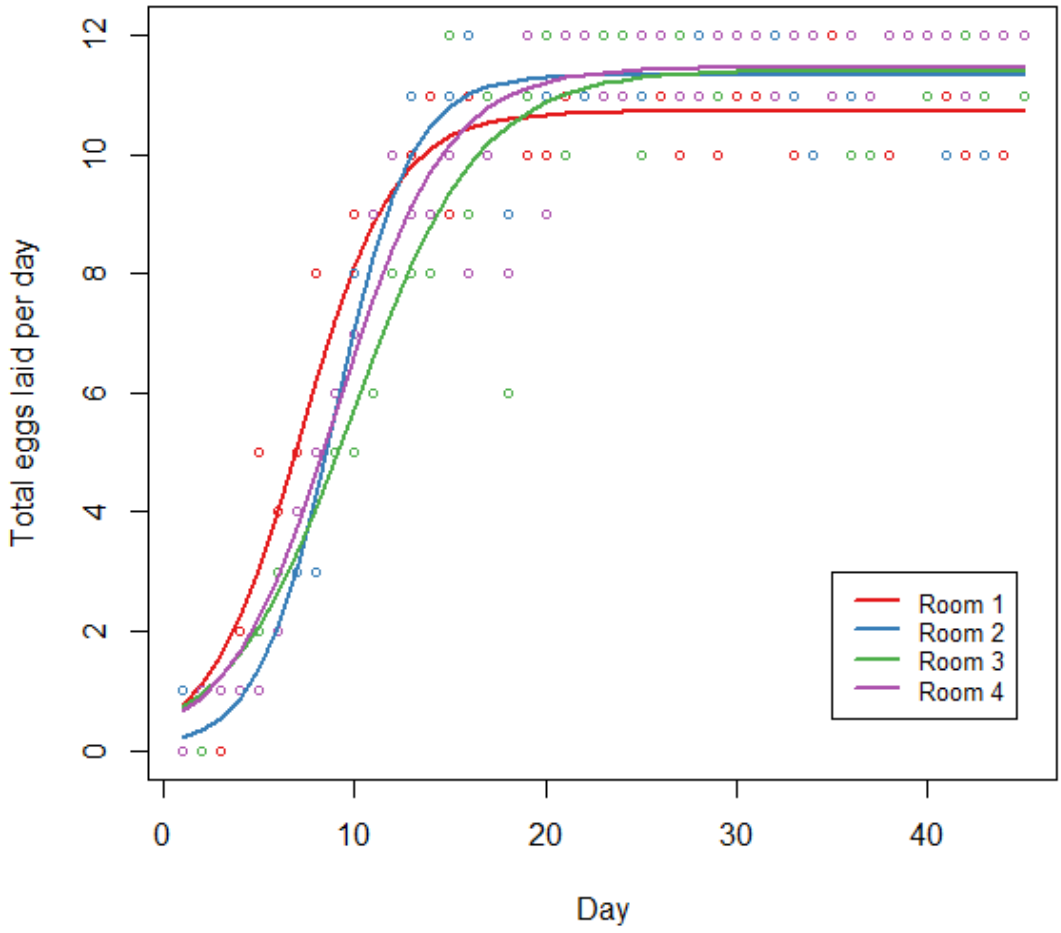

Figure 4.16 - Sigmoid curves for total eggs laid per day in each room from the day of the first egg (day 0) until the end of the UCMS period (day 45). Birds in room 1 and 3 were exposed to UCMS treatments, while birds in rooms 2 and 4 were controls.

The number of eggs per day at which production stabilised in each room, as measured by the *a* estimate was very similar for rooms 2, 3, and 4, ranging between 11.34 and 11.47 eggs per day. Egg production in room 1 stabilised at a slightly lower level of 10.73 eggs per day.

4.4.9. Hippocampal gene expression

There was a significant three-way interaction between stress, rearing, and hippocampal subregion on DCX expression ($F_{1,41} = 9.44$, $p = 0.004$). Post hoc pairwise comparisons show that in the caudal HF, there was significantly higher DCX in UCMS than control birds (Figure 4.17), but only in those reared with a dark brooder ($p = 0.044$). In conventional birds, there tended to be slightly lower DCX expression in the caudal HF of UCMS vs control birds, but this did not reach the threshold for significance ($p = 0.211$). There was no significant effect of UCMS on DCX expression in the rostral hippocampus in dark brooder reared ($p = 0.511$) or conventionally reared birds ($p = 0.452$).

There was no effect of rearing in the rostral HF in control (p = 0.507) or UCMS birds (p = 0.456). In UCMS birds, those reared with a dark brooder tend to have slightly higher DCX expression in the caudal hippocampus than those reared conventionally, however this effect was not significant (p = 0.071). There was no effect of rearing on DCX expression in the caudal HF of conventionally reared birds (p = 0.148).

There was significantly higher DCX expression in the caudal HF than rostral HF in control conventional (p = 0.041) and UCMS dark brooder birds (p = 0.006), but no significant difference between the two hippocampal subregions in UCMS conventional (p = 0.559) or control dark brooder birds (p = 0.490).

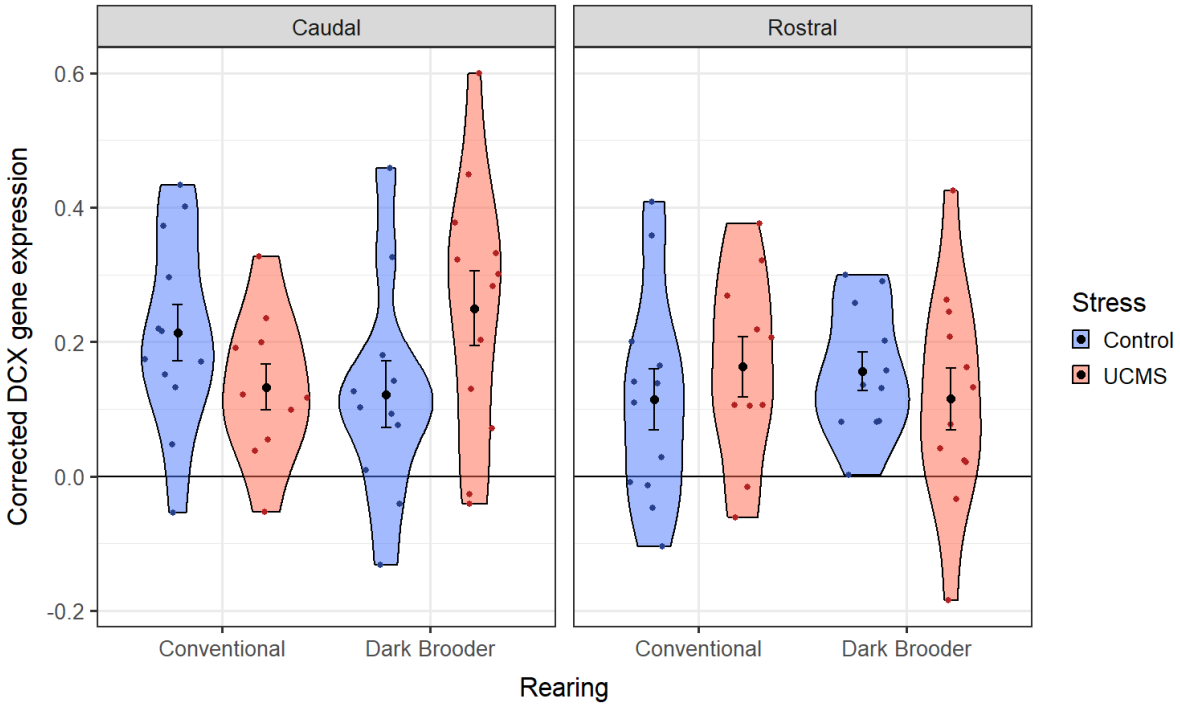

Figure 4.17 – Distributions of expression of DCX mRNA in the caudal and rostral hippocampal formation of control and UCMS birds reared conventionally or with a dark brooder. DCX expression in each individual sample was corrected for LBR expression in the same sample. Values are log_{10} ratios, transformed by adding a coefficient of 1. Black points and error bars represent mean ±1SE corrected expression of DCX mRNA.

There was no main effect of stress ($F_{1,41}$ = 0.13, p = 0.719), rearing ($F_{1,41}$ = 0.02, p = 0.897), or subregion ($F_{1,41}$ = 3.05, p = 0.088) on DCX expression. There were also no two-way interactions between stress and rearing ($F_{1,41}$ = 0.63, p = 0.432), stress and subregion ($F_{1,41}$ = 0.17, p = 0.682), or rearing and subregion ($F_{1,41}$ = 0.11, p = 0.741).

There was no main effect of stress ($F_{1,41}$ = 1.19, p = 0.282) or rearing ($F_{1,41}$ = 1.39, p = 0.245) on BDNF expression, though there was significantly higher BDNF expression in the rostral HF than

the caudal HF ($F_{1,41}$ = 29.79, p < 0.001) (Figure 4.18). The interaction between stress and rearing on BDNF expression did not reach the threshold for significance ($F_{1,41}$ = 3.89, p = 0.055).

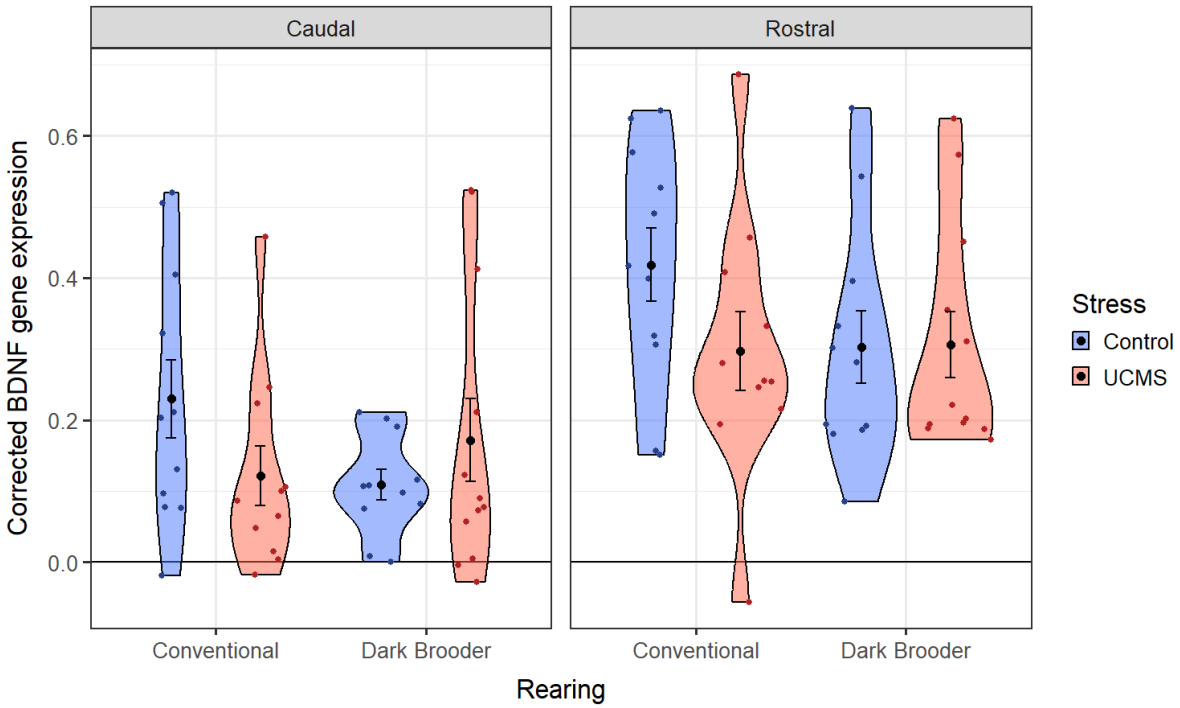

Figure 4.18 - Distributions of expression of BDNF mRNA in the caudal and rostral hippocampal formation of control and UCMS birds reared conventionally or with a dark brooder. BDNF expression in each individual sample was corrected for LBR expression in the same sample. Values are log_{10} ratios, transformed by adding a coefficient of 1. Black points and error bars represent mean ±1SE corrected expression of BDNF mRNA.

There was no two-way interaction between stress and subregion ($F_{1,41}$ = 0.32, p = 0.576) or between rearing and subregion ($F_{1,41}$ = 0.08, p = 0.775). There was no three-way interaction between stress, rearing, and subregion ($F_{1,41}$ = 0.14, p = 0.714) on expression of BDNF.

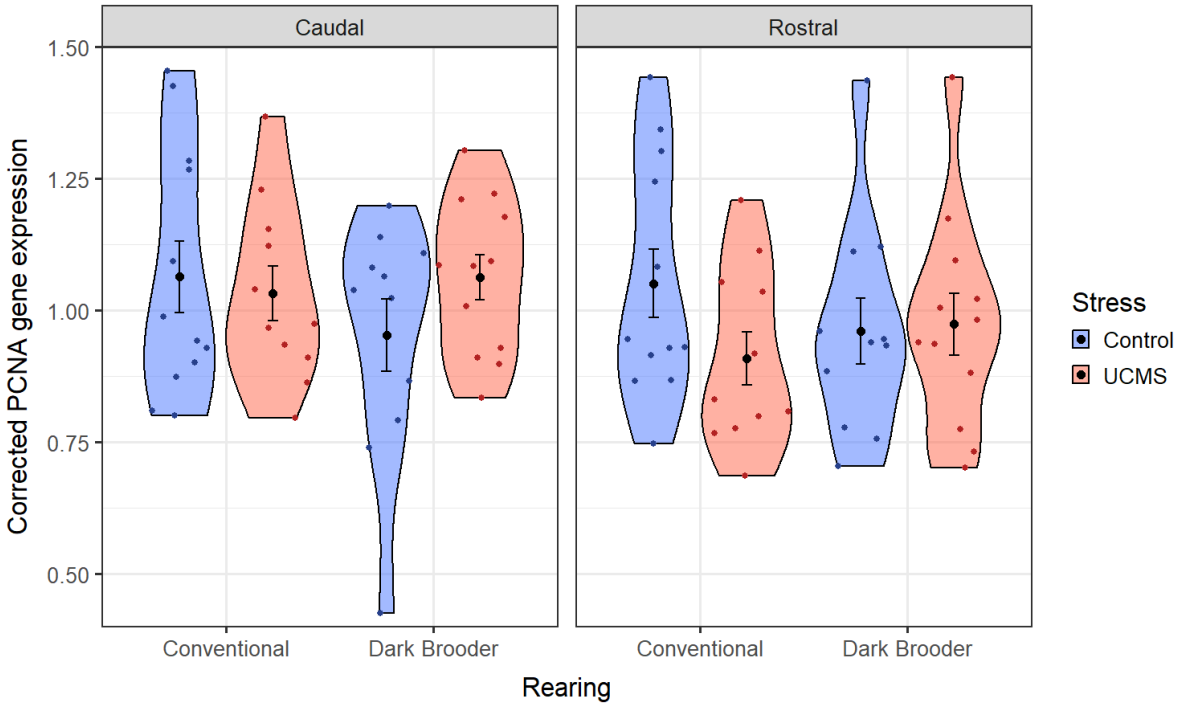

Figure 4.19 - Distributions of expression of PCNA mRNA in the caudal and rostral hippocampal formation of control and UCMS birds reared conventionally or with a dark brooder. PCNA expression in each individual sample was corrected for LBR expression in the same sample. Values are log_{10} ratios, transformed by adding a coefficient of 1. Black points and error bars represent mean ±1SE corrected expression of PCNA mRNA.

There was no significant main effect of stress ($F_{1,42}$ = 0.06, p = 0.807), rearing ($F_{1,42}$ = 0.27, p = 0.607), or hippocampal subregion ($F_{1,42}$ = 3.36, p = 0.074) on PCNA expression (Figure 4.19). There were also no two-way interactions between stress and rearing ($F_{1,42}$ = 2.10, p = 0.155), stress and subregion ($F_{1,42}$ = 3.10, p = 0.085), or rearing and subregion ($F_{1,42}$ = 0.22, p = 0.644). There was no three-way interaction between stress, rearing and subregion ($F_{1,42}$ = 0.01, p = 0.912).

4.5. Discussion

Our UCMS protocol was validated by the results of the TI tests, which showed an increase in fear-related behaviour from baseline only in birds which had been subjected to UCMS. However, there was no main effect of UCMS on any outcomes in the NOT, blood plasma CORT or expression of genes related to hippocampal plasticity. Also, there was no main effect of rearing on gene expression, plasma CORT, or TI. However in the NOT, dark brooder reared birds spent significantly more time close to the door of the testing arena than conventionally reared birds, and the interaction between the effects of rearing and circle in the NOT suggest

that dark brooder birds may have been less anxious when exploring the areas of the arena closest to the novel object.

Though the predicted main effects of UCMS or rearing alone were not observed for some of our outcomes, it appears that the effect of UCMS on some outcomes was dependent on the condition in which birds had been reared. In the caudal HF of conventionally reared birds, there was a slight decrease in DCX expression in response to UCMS which, while not a statistically significant effect, was in the predicted direction. However, in birds reared with a dark brooder, the response was in the opposite direction to what would typically be expected, with significantly increased DCX expression in the caudal HF in UCMS birds. Responses to acute stress in adulthood were also dependent on both chronic stress and rearing condition, with elevated plasma CORT after restraint in a bag only observed in conventionally reared birds which were not exposed to UCMS.

These results suggest that rearing with a dark brooder in the first three weeks of life affects the brain, causing effects on the stress response which persist into adulthood. This supports our main hypothesis, that birds reared with a dark brooder would be more resilient to chronic stress than those reared conventionally. The full results are discussed in more detail below.

4.5.1. Effects of UCMS

TI has been validated as a test for fearfulness in chickens by many previous studies (Forkman *et al.*, 2007), and it is believed that chronic stress makes birds more fearful. Longer TI durations were observed at week 6 than at pre-treatment overall, which appears to suggest greater fear across all groups at week 6. However, from the post-hoc pairwise comparisons following the significant interaction between time-point and stress, it is clear that the overall increased TI duration at week 6 was driven exclusively by the UCMS birds, which had greater TI durations at week 6 than pre-treatment while the control birds did not. These findings support the efficacy of our UCMS protocol, because UCMS caused an increase in fear-related behaviour from baseline that was not observed in control birds.

As observed in the TI tests, there was also no main effect of UCMS on any of the behavioural outcomes from the NOT, while time-point had a significant main effect on all outcomes (except freezing, though this was very close to the threshold for significance). At week 7, the responses of all birds, including controls, were in the direction that was expected in response to chronic stress for latency to first movement, time spent near the door, and time spent in circles close to the novel object. It is possible that all birds were sufficiently stressed by the

housing conditions, independent of experimentally induced stress, that they all exhibited anxiety-related behaviours in an unfamiliar situation, though this explanation is inconsistent with the findings from TI testing. Instead, it is more likely that our chosen behavioural outcomes for the NOTs were not sensitive enough to detect the effects of UCMS at the later time-point, when greater anxiety-related behaviours were expected in the UCMS birds.

The only significant interaction between time-point and stress was on time spent moving during the NOTs. A difference in time spent moving between the two time-points was only observed in the UCMS birds, though this was in the opposite direction to what was predicted. It was expected that birds would have lower movement during the NOTs after they were exposed to chronic stress, because more anxious or fearful birds are less likely to explore an environment containing a novel object (Jones, 1996). However, time spent moving increased after exposure to UCMS, suggesting our hypothesised effects of UCMS on movement were not correct. Contrary to our prediction, Zimmerman *et al.* (2011) found that hens displayed increased steps and head movements when anticipating a negative event. Exploration may not have been the only motivation for increased locomotion during the tests, and if UCMS induced anxiety, the birds may have moved around more to look for opportunities to escape or the check for predators. Perhaps "moving" could have been broken into separate behaviours to distinguish exploration from escape attempts and vigilance behaviours, allowing us to better understand the motivation for the increased movement during the second NOT.

Freezing is a common behavioural response in birds which makes them less detectable to potential predators (Gallup and Suarez, 1980) and is often used as an indicator of anxiety. In the current study, I was careful to distinguish "freezing" from "still", in which birds may have been performing other behaviours while standing still in one place. In many individuals, no freezing behaviours were recorded throughout the tests and time spent freezing was not significantly affected by any of the factors I investigated. The birds spent less time in in the 60cm and 30cm circles at week 7 than at pre-treatment, which is consistent with other outcomes suggesting that the birds were generally more anxious during the second NOT. However, there was no interaction between time-point and stress, so the decreased time spent close to the object seems to be independent of the effect of UCMS.

Decreased baseline CORT in response to chronic stimulation of the HPA axis by UCMS was expected, as was observed by Gualtieri *et al.* (2019) in a comparable study. Though there was

a main effect of week, with CORT levels decreased from baseline after 4 weeks of the UCMS period, these differences were observed across all groups and were not significantly affected by UCMS as predicted.

There was no effect of UCMS on body mass before or after onset of lay, or on the day of the dissections. This suggests that the chronic stress induced in UCMS birds was not severe enough to affect the rate of growth. Though the rate of growth decreased after 19 weeks of age (Figure 4.13), age alone predicted body mass both before and after onset of lay in all groups. After correction for final body mass, I expected to see smaller spleens in UCMS birds as found in Gualtieri *et al.* (2019), though that was a weak effect. I found no effect of chronic stress on spleen mass, suggesting no effects on immune function. Spleens are known to shrink with chronic CORT or ACTH administration (Davison, Freeman and Rea, 1985). Though exogenous CORT was not administered in this study, there was no significant main effect of UCMS on measured CORT. If it is true that CORT is moderating spleen mass, this may explain why the expected effect in the spleen was not observed between the groups.

The first egg was laid by a control bird, at 18 weeks and 4 days of age, with no eggs laid by UCMS birds until two days later. The stressor in the UCMS rooms on the day that the control birds started laying was “short day”, in which the birds only received a total of 4 hours light within a 24-hour period. This may have interfered with the circadian rhythm of lay in UCMS birds, contributing to the slightly later onset of lay. However, this slightly later onset in UCMS birds does not seem to have a large effect on early egg production. Chi-squared tests conducted on the total number eggs laid in each room during the first 10 days since the first egg was laid indicate that there was no overall effect of UCMS on egg production during this period, in which the greatest amount of variation was expected between birds from different treatment groups.

Because eggs were counted per room rather than per individual, statistical analyses such as chi-squared tests may lack sufficient statistical power to detect any differences between treatments or between rooms. Instead, it was more appropriate to use the parameter estimates from a sigmoid curve for each room to describe trends in egg production. Here, egg data collected from the onset of lay until the end of the UCMS period (45 days total) was used to fit the sigmoid curves, which allowed an estimate of when the number of eggs laid per room stabilised, which would not have been possible using the data from the first 10 days only.

The parameter estimates do not appear to show a difference in egg production between the UCMS and control rooms. The curve for one of the UCMS rooms (room 1) had an earlier inflection point than the control rooms, and the eggs laid in this room stabilised at a slightly lower level than in the control rooms. This slightly reduced egg production may appear to support the hypothesis that UCMS decreases egg production, however, the curve for the other UCMS room (room 3) was very similar to the curves for the control rooms in both *a* and *m* parameter estimates. This suggests that the chronic stress experienced by birds in this room was not sufficient to negatively affect egg production. If any differences in egg production do exist, they were more likely to be driven by a room effect than by chronic stress, though this was not clear from the results.

There was no main effect of chronic stress on the expression of DCX, BDNF, or PCNA, and no interaction between stress and subregion of HF on any of the genes. This was unexpected, because a similar UCMS protocol (Gualtieri *et al.*, 2019) has previously decreased hippocampal plasticity at the caudal pole of the HF. In Gualtieri *et al.* (2019), all birds were reared conventionally and the effect was only observed in the caudal HF. In all three plasticity genes, the effect of UCMS in the caudal HF of conventional birds was in the expected direction, although the differences were not statistically significant. It is likely that the effect of UCMS is dependent on both the subregion of the HF and the environment in which the birds were reared, as discussed in detail below.

4.5.2. Effects of dark brooders

My predictions were not about the main effects of rearing specifically, but about the way in which rearing affected the effects of UCMS. Nevertheless, there were some interesting main effects and other interactions involving rearing. There was no main effect on TI duration, and time spent near the door was the only outcome from NOT on which the main effect of rearing was significant. Time spent near the door is unique to our study rather than a standard outcome for NOT, but it may provide rationale for a future study to investigate effects of dark brooders on the motivation of birds to be with conspecifics. The birds were able to see their conspecifics through the mesh in the door between the pens, so the increased time spent by the door by dark brooder reared birds may indicate a stronger motivation for social reinstatement, as was observed by de Jong *et al.* (2022). Dark brooders provide a safe space for chicks to hide during early life, and they are less likely to experience aggression from conspecifics. This may cause increased social behaviour, which persists into adulthood.

There was no significant main effect of rearing on any other outcomes from the NOTs, which is inconsistent with the findings of previous studies in which similar tests were used. For example, Riber and Guzman (2016) found that dark brooder birds had a shorter latency to first movement in an open field test and moved closer to a novel object than birds reared without a brooder. Unlike our birds, those tested by Riber and Guzman (2016) were allocated to 22 pens (11 dark brooder, 11 control) at one day of age, and NOTs were conducted in the same pens where they were reared. In each of our pens, dark brooder and conventionally reared birds were mixed, which may have affected their behaviour. Also, our birds were tested individually. Their open field tests were conducted individually, with 10 birds selected from each pen, however their NOTs were conducted at group-level within pens.

The interaction between the effects of rearing and circle in the NOT show that while conventionally reared birds spent more time in the 60cm circle than the 30cm circle, dark brooder birds explored these two circles equally (after correction for the area of the circles), suggesting that they may have been willing to investigate the novel object and therefore may have been less anxious. This is a tentative conclusion, because there was no overall effect of rearing on time spent in 30cm circle, as would be expected if rearing affected willingness to investigate the novel object.

4.5.3. Effects of rearing on responses to UCMS

Although the predicted main effect of UCMS on TI duration was observed, the TI results do not fully support our hypothesis, because the increase in TI durations were not smaller or non-existent in dark brooder reared birds than conventionally reared birds at either time-point or in either stress condition. This is inconsistent with previous studies such as Riber and Guzman (2016), in which dark brooder reared birds had shorter TI durations at all ages tested, and does not provide evidence that dark brooders conferred resilience to chronic stress in the current study. There were no significant interactions between rearing and stress or time-point on any outcomes from the NOT, so overall, the results from the NOTs also do not support our main hypothesis about the effect of brooders on stress resilience.

The results from blood plasma CORT suggest that responses to acute stress in adulthood are dependent on interactions between adult chronic stress and early-life experiences. Rearing was involved in an interesting three-way interaction with UCMS and restraint, which may aid our understanding of the relationship between the rearing environment and both chronic and acute stress later in life. In control birds that were reared conventionally, the expected

response to acute stress after restraint in a bag was observed: significantly elevated CORT from baseline. This typical response was not observed in UCMS birds, or in dark brooder birds. These effects were not additive, as evidenced by the dark brooder reared UCMS birds, and UCMS and dark brooder rearing may diminish the CORT response to acute stress by different mechanisms. Birds exposed to chronic stress may not have elevated CORT responses to restraint due to chronic stimulation of the HPA axis, while dark brooder birds may be displaying resilience to the effects of stress, though further study is needed to understand the mechanism by which this resilience develops.

The hippocampal gene expression results provide the strongest support for the hypothesis about the effects of the rearing environment on adult responses to chronic stress. In conventionally reared birds, UCMS tended to cause slightly lower DCX expression in the caudal HF. Though this difference was not significant, it is in the direction that would be expected because the density of DCX^+ cells has been shown to decrease in the caudal HF in response to chronic stressors (Gualtieri *et al.*, 2019; Armstrong *et al.*, 2020a). However, in dark brooder birds, UCMS significantly increased DCX expression in the caudal HF, which is in the opposite direction to what was expected. There were no main effects of stress or rearing, so this effect of chronic stress on DCX in the caudal HF appears to be dependent on the rearing environment.

It appears that rearing with a dark brooder changes the way the brain responds to stress later in life, though further study is required to elucidate the mechanism that causes these contrasting effects of stress on DCX expression between the two rearing groups. One possible explanation could be that the effect of chronic stress on DCX expression follows an inverted U curve. Previous studies have proposed a similar relationship between stress and hippocampal function, for example, Conrad, Lupien and McEwen (1999) found that treatment with low levels of CORT increased hippocampal spatial memory in rats, but higher levels of CORT administration impaired performance in spatial memory tests. Similarly, Kirby *et al.* (2013) found that adult hippocampal neurogenesis may follow an inverted U curve, with acute stress increasing cell proliferation in the hippocampus of rats while chronic stress decreased cell proliferation. These studies both proposed inverted U curve shaped relationships between stress and hippocampal function in the dorsal HF, not the ventral HF which is most sensitive to the effects of stress in mammals. In the current study, there appears to be a similar effect in the caudal HF, homologous to the mammalian ventral HF. It could be possible that plasticity

(as measured by DCX gene expression) initially increases in response to UCMS, until a threshold is reached at which point plasticity is decreased by UCMS in in a similar pattern to what was observed in the rats after exposure to chronic stress. It may be that the conventionally reared birds already experienced elevated stress in early life, shifting them towards the inflection point of the inverted U curve at which cell plasticity begins to decline after reaching a threshold level of chronic stress. The dark brooder reared birds, however, may be more naïve to chronic stress, and therefore their brains respond to the UCMS challenge by increasing neural plasticity in order to moderate their stress response. If this were true, it would support our hypothesis about dark brooder rearing conferring resilience to stress later in life. Therefore, implementation of dark brooders on rearing farms may have positive welfare implications that persist into adulthood, such as increased ability to cope with common stressors experienced by commercially housed hens, with higher levels of chronic stress needed throughout life before adult birds would enter the depression-like state observed in conventionally reared birds in this and previous studies (such as Robertson *et al.* (2017); Gualtieri *et al.* (2019); Armstrong *et al.* (2020a)).

Expression of BDNF followed a similar pattern to that of DCX, though the effects were more subtle. In conventionally reared birds, again, there was a decrease in hippocampal plasticity (as measured by BDNF expression), though this was not specific to the caudal subregion. This decreased plasticity in response to chronic stress was not observed in dark brooder birds, again supporting the hypothesis that dark brooders confer resilience, though not as strongly as with the findings from DCX. There were no significant findings from PCNA in either subregion of the hippocampus, which may be due to the role of PCNA in promoting cell proliferation in all cell types, rather than exclusively in the immature neurons that would be sensitive to chronic stress.

4.5.4. Study limitations

While the observers made every effort to treat the birds consistently during induction during TI testing, the tests were conducted in an empty pen adjacent to the birds' home pens and it is possible that birds were disturbed by sound from the other birds, which may have affected TI times. To avoid this problem in future studies, it may be better to carry the birds to a different room for testing.

NOT may also have been improved by using different objects, or by using an alternative type of NOT such as that used by Fraisse and Cockrem (2006), in which a novel object was placed

in a food trough in front of the cage. In our tests, the birds did not have access to food, which may have affected the motivation to interact with the object. While our findings about the effect of rearing on time spent near the door during NOT was interesting, the visibility of conspecifics through the door between the pens was a limitation of our study design, and may have been a distraction during the tests, affecting the results of the other outcome measures. In future, it should be ensured that there is no visual distraction to the birds during tests. Also, the area close to the door was not explicitly defined for the observer of the videos in the same way that the circles were. The observer standardised this area as between the door, the nearest corner of the mat, and the nearest wall of the arena, however these lines were difficult for the observer to define and it would have been better to mark this area in the arena for more consistent scoring.

Though there was no effect of chronic stress or rearing on body mass, there appears to be some variation between the groups, especially at 24 weeks of age with a slightly lower body mass in UCMS birds compared to controls. This may be explained by the timing of the weighing during week 24, as UCMS birds were weighed after they were released from the "pack in boxes" stressor during which they did not have access to food for 3 hours. It is possible that the UCMS birds may have had less food stored in their crops during weighing, resulting in artificially lower recorded body mass than in the control birds who had continuous access to food on the day of weighing. In future, this error could be avoided by weighing the birds at a consistent time of day, before any stressors were applied.

Unfortunately, the design of the study did not allow us to investigate the effects of rearing on egg production because each room contained a mixed group of dark brooder reared and conventionally reared birds, and the experimental unit for egg counts was the room rather than individual birds. In a future study, it may be interesting to investigate whether early life experience can confer resilience to any loss in egg production which may be caused by chronic stress, though the current study does not provide evidence for such an effect.

4.5.5. Conclusions

In the current study, measures from the brain, blood plasma CORT, and behavioural responses to chronic stress were investigated in order to understand how providing dark brooders in the first three weeks of life can affect responses to chronic stress during adulthood. The results from the DCX expression provide our strongest support for our hypothesis that birds reared with a dark brooder are more resilient to chronic stress than those reared conventionally.

The findings from blood plasma CORT analysis may also support our hypothesis, as control dark brooder birds did not display the typical elevated CORT response to acute stress as was observed in the conventionally reared control birds. However, the results from the behavioural tests were less clear, and did not support our hypothesis as there were no significant main effects or interactions with rearing on any behavioural outcomes that were measured.

Our findings provide a basis for further investigation of the mechanism by which the rearing environment can influence later responses to chronic stress. The findings from hippocampal gene expression and blood plasma CORT seem to suggest welfare benefits of dark brooders for birds exposed to experimentally induced chronic stress, though welfare implications cannot be asserted too strongly because strong behavioural responses to chronic stress were not induced. Though not all the measured outcomes supported the main hypothesis that dark brooder rearing would confer resilience to chronic stress in adulthood, the current study did not find any way in which dark brooder rearing causes harm, and is consistent with the findings from other recent studies which call for the wider implementation of dark brooders on commercial rearing farms.

Chapter 5. General discussion

5.1. Summary of key findings

In this thesis, I used hippocampal plasticity to measure chronic stress caused by housing systems and related health conditions, and to investigate the effects of dark brooders as an early life intervention which may confer resilience to stress in adulthood. The aims outlined in Chapter 1 were addressed in three studies, the key results of which are summarised below. Here, I will integrate the findings of these three studies and discuss the overall findings of this thesis, and their implications for welfare and methodology for future studies. I will also propose possible future studies which may address questions that remain unanswered.

In Chapter 2, I addressed the first aim of the project, which was to investigate whether differences in environmental complexity cause chronic stress. Between birds moved from a flat-deck rearing site to a flat-deck laying system, and birds moved from the same rearing site to a multi-tier aviary, there was no difference in hippocampal plasticity in either the caudal or rostral hippocampal formation (HF). Not only does this suggest that there was no difference in the level of chronic stress experienced between hens housed in these two systems with differing environmental complexity, but also that any difference in spatial stimulation caused by these two housing deigns was not great enough to be detected using our measure of hippocampal plasticity.

In addition to comparing housing designs directly, I aimed to use hippocampal plasticity to measure the potentially stressful effects of a common health condition which is believed to be caused by the housing system, namely, footpad dermatitis (FPD). This aim was addressed in Chapter 3, in which I found evidence partially supporting the hypothesis that FPD is a chronic stressor, though the effect of FPD on hippocampal plasticity in adult hens was dependent on the environment in which they were reared. While hippocampal plasticity tended to decrease across the whole HF in response to FPD in conventionally reared birds, it tended to increase in birds reared with a dark brooder. This may suggest that dark brooders play a role in conferring stress resilience to hens which persists throughout life, though the study presented in chapter 3 was lacking the statistical power needed to confirm this.

In Chapter 4, I further investigated the effects of dark brooders on resilience to stress during adulthood. Chronic stress was induced experimentally using an unpredictable chronic mild stress (UCMS) paradigm, which was found to increase tonic immobility (TI) durations from a

baseline recorded before UCMS treatment. UCMS tended to cause a decrease in hippocampal plasticity, as measured by expression of doublecortin (DCX) in the caudal HF of conventionally reared birds. Similar to the findings with FPD in Chapter 3, the effect of UCMS on hippocampal plasticity was in the opposite direction in dark brooder birds. Here, hippocampal plasticity significantly increased in the caudal HF in dark brooder birds when challenged with UCMS. In addition to measuring hippocampal plasticity, corticosterone (CORT) titres were also measured in Chapter 4. CORT was found to be elevated from baseline following an acute stressor (restraint in a bag), but only in conventionally reared birds which were not exposed to UCMS. This suggests that responses to acute stress in adulthood are dependent on both chronic stress and the rearing environment, though the effects were not additive.

5.2. Effects of putative stressors on conventionally reared hens

5.2.1. Housing and related conditions

In the majority of previous studies investigating chronic stressors in hens, subjects have been reared without a dark brooder, except in cases where the effect of dark brooder rearing was being investigated specifically. Chapter 2 was the only study in this thesis in which all birds were reared conventionally, as the focus of that study was the transition to adult systems rather than any specific aspects of the rearing environment. Also, healthy birds were used in this study, selected to be of average body condition and without keel bone damage (KBD) to ensure that only the effects of environmental complexity were being measured. There was no effect of the adult environment on hippocampal plasticity, which means the findings from Chapter 2 do not support the hypothesis that hens would experience higher spatial stimulation and lower levels of chronic stress when moved to a higher complexity laying system compared to hens which were moved to a lower complexity laying system from the same rearing site. This could have been expected due to no difference in hippocampal plasticity between birds in furnished cages and multi-tier aviaries (Armstrong *et al.*, 2022), which also suggests that the complexity of the environment itself does not have a main effect on stress.

In the remaining two studies in this thesis, effects of putative stressors were measured in both conventionally and dark brooder reared hens, and significant interactions were found between adult response to stressors (as measured by hippocampal plasticity) and the rearing environment. In Chapter 3, post hoc pairwise comparisons (following the significant interaction effect in the main analysis which compared severe FPD compared to controls)

showed a trend towards a decrease in hippocampal plasticity in conventionally reared birds only. Post hoc pairwise comparisons were not possible following the analysis which treated cumulative FPD score (treated a continuous variable), however, Figure 3.5 shows that there was also a trend towards decreasing DCX^+ density across the whole HF as cumulative FPD severity increased. Though neither of these trends were statistically significant, they are in the direction in which we would expect to see in response to chronic stress based on previous studies which have used similar methods to quantify the effects of chronic stressors (Gualtieri *et al.*, 2019; Armstrong *et al.*, 2020a; Armstrong *et al.*, 2022).

There was also a significant main effect of KBD in the analysis comparing FPD birds to controls, though the effect was in the opposite direction to what was predicted based on findings by Armstrong *et al.* (2020a), and there was no effect of KBD when treating cumulative FPD score as continuous. Though KBD was not an intentional aspect of that study, it is thought to be related to housing conditions (Sandilands, Moinard and Sparks, 2009; Harlander-Matauschek *et al.*, 2015). Therefore, it is important to consider KBD as a factor in this study.

5.2.2. Comparison with UCMS

UCMS has been validated as a chronic stressor in chickens by previous studies, specifically decreasing hippocampal plasticity as measured by density of DCX^+ cells in the caudal HF (Gualtieri *et al.*, 2019) and neuronal remodelling (Tamta *et al.*, 2023; Arya *et al.*, 2024). The specific UCMS protocol used in Chapter 4 was found to induce fear responses, measured by increased duration of TI from baseline following UCMS, suggesting it successfully induced chronic stress.

A similar pattern in DCX gene expression was observed in response to UCMS as was observed in DCX immunoreactivity in response to FPD in Chapter 3. This similarity in the effects on hippocampal plasticity may add weight to the argument that FPD was also chronically stressful, though there were many differences between those two studies that are important to consider. Firstly, the effect of UCMS was specific to the caudal HF, as is expected in response to stress (Smulders, 2017), and as was previously found in response to UCMS (Gualtieri *et al.*, 2019). However, the effect of FPD was observed across the whole HF. The birds in Chapter 3 were also much older (70 weeks of age) at the time when brains were sampled than the birds in chapter 4 (25 weeks of age), and hippocampal plasticity can decline with age. Statistical power was low in Chapter 3 due to a small sample size and multiple between subject factors (FPD, rearing, KBD) included in the analysis. Also, the effects of FPD may have been

confounded by KBD due to uneven numbers of birds with KBD between groups. These may all be reasons why the effects of stressors on hippocampal plasticity (and differences in the direction of the responses to stress between the two rearing environments) were more pronounced in Chapter 4 than in Chapter 3. A study with a higher sample size and without the influence of KBD (confirmed by palpation before selection of birds) would be required to accurately quantify differences in effect size between UCMS and FPD.

5.2.3. Welfare implications

In commercially reared hens, specific health conditions which are caused or exacerbated by the housing environment such as FPD, KBD, both in my Chapter 3 and Armstrong *et al.* (2020a), and poor body condition (Armstrong *et al.*, 2022) may have greater impact on welfare than the design inside the barn. Complexity of the housing design may affect welfare indirectly by causing stressful health conditions rather than by causing chronic stress directly itself in birds with no pathological symptoms. In future studies, it may be more helpful to address welfare impacts of health issues directly rather than as consequences of housing, as FPD (Wang, Ekstrand and Svedberg, 1998; Pagazaurtundua and Warriss, 2006; Shepherd and Fairchild, 2010) and KBD (Casey-Trott *et al.*, 2015; Heerkens, 2016) are prevalent in all systems.

5.3. Effects of dark brooder rearing

5.3.1. Responses to chronic stress are dependent on the rearing environment

In both Chapters 3 and 4, the responses to chronic stressors were in opposite directions in dark brooder reared birds than in conventionally reared birds. Although the effect of FPD was not statistically significant and was not specific to the caudal HF, the direction of the effect provides support for the hypothesis that rearing chicks with a dark brooder confers resilience to stress during adulthood. The effect was even more pronounced in Chapter 4, with a statistically significant increase in DCX expression in the caudal HF of dark brooder reared UCMS hens compared to controls. Not only does it support the main hypothesis of the study, but it goes beyond the prediction that the effect of experimentally induced chronic stress on hippocampal plasticity would be smaller in adult hens reared with a dark brooder compared to those reared conventionally.

The more pronounced effect in Chapter 4 may be due to multiple factors which were different to Chapter 3, including the younger age of the birds, the lack of possible confounding effects of KBD, differences in management (both in experimental pens and in the rearing sites), and higher statistical power due to more birds per group. To accurately compare the effect sizes

of FPD and UCMS, the FPD study would need to be repeated with birds of a similar age, which did not have any pathologies other than FPD. These differences aside, the similarity of the pattern of the effects of dark brooders between the two studies suggests a consistent effect of dark brooder rearing on responses to stress during adulthood.

Both chapters support the theory of an inverted U shaped relationship between chronic stress and hippocampal plasticity, similar to theories which have been previously proposed as explanations of the relationship between chronic stress and hippocampal function in rats (Conrad, Lupien and McEwen, 1999; Kirby *et al.*, 2013). While the hippocampus of conventionally reared birds appears to respond to chronic stressors in a similar pattern to mammals which are suffering from depression-like states (declining AHN, after reaching a threshold at which addition of new neurons to the hippocampus is no longer beneficial for regulating stress), dark brooder reared birds respond differently, and hippocampal plasticity appears to be on an upwards trajectory towards that threshold, but not yet past it. In a review of studies on the effects of stress and environmental enrichment on emotional regulatory circuits in rats, Smail *et al.* (2020) suggest that some housing conditions such as environmental enrichment may induce a mild and beneficial type of stress which allows the brain to adapt to a stressful environment, thereby conferring stress resilience. Individuals which are not exposed to mild "stressors" such as these (e.g. rats housed in single cages or an unenriched environment) may have impaired stress resilience due to the inability to develop a healthy stress response early in life. It is unclear whether conventionally reared chicks are missing these positive experiences, which may be provided by maternal care in a natural setting, and are required to acquire a healthy stress response, or if they experience too much stress early in life, which shifts them close to the inflection point of the inverted U curve so hippocampal plasticity begins to decline after exposure to chronic stressors. Simulation of maternal care by a dark brooder may provide chicks with optimal opportunities to increase neural plasticity while they are naïve to chronic stressors early in life, and therefore they add new neurons to the HF when faced with chronic stressors. This is currently speculation, and further study is needed to confirm these proposed mechanisms.

5.3.2. Implications for welfare and industry

The findings of this thesis add to previous research by indicating that the provision of dark brooder to layer chicks, even for only the first three weeks of life, changes the way the brain responds to stressors during adulthood. My findings support the hypothesis that dark brooder

rearing confers long term stress resilience effects, and may persist until at least 70 weeks of age according to the results of Chapter 3. These findings are somewhat similar to the well-established persistent effects of maternal care in mammals, such as maternal licking and grooming behaviours in rats (Liu *et al.*, 1997; Champagne *et al.*, 2008; Nguyen *et al.*, 2015; Nguyen *et al.*, 2018).

Despite numerous behavioural studies on the benefits of dark brooders in the reduction of harmful behaviours such as injurious pecking, the poultry industry has been slow to implement dark brooders on rearing sites (Sirovnik and Riber, 2022). Further known benefits include more economical heating during the first few weeks of life, compared to whole house heating (Edgar *et al.*, 2016). The findings of this thesis support the call for implementing dark brooders on commercial rearing sites more widely.

5.4. Hippocampal plasticity as a measure of chronic stress

5.4.1. Sensitivity of hippocampal plasticity to subtle stressors

It was expected that environmental complexity would be a less severe stressor than conditions investigated in previous studies such as severe KBD or experimentally induced UCMS. Therefore, in addition to investigating the severity of environmental complexity as a stressor, Chapter 2 also addressed the aim to evaluate the efficacy of hippocampal plasticity of a biomarker of more subtle stressors in laying hens. I found that quantifying DCX^+ cell density was not sensitive enough to detect more subtle differences in either chronic stress or spatial stimulation, even in two environments which were apparently different in complexity. As previously stated in Chapter 2, access to the range for at least 4 weeks in both groups may be responsible for diminishing the effects of environmental complexity on both spatial stimulation and stress. Repeating the study inside the barn only, or in experimental pens with no outdoor access may help to elucidate whether our method was less sensitive than expected, or if this was caused by access to the range.

5.4.2. Support for functional gradient

There was conflict between studies in this thesis in support for the proposed functional specialisation along the rostro-caudal axis of the avian HF (Smulders, 2017). The three-way effect between UCMS, rearing, and hippocampal subregion on DCX gene expression supports the proposed functional gradient because the effects of UCMS and rearing were only significant in the caudal HF, which is believed to be homologous to the mammalian ventral dentate gyrus in its sensitivity to chronic stress. There was no significant effect of UCMS or

rearing on DCX expression in the rostral HF, which was expected because of the proposed role of the rostral HF in spatial memory and cognition rather than mediation of stress. However, there were no significant interactions involving hippocampal subregion in Chapter 3. The effect of FPD appears to be more pronounced in caudal HF than the rostral HF, this is not sufficient evidence to add weight to the hypothesis of functional specialisation and is more consistent with the effect of KBD in Armstrong *et al.* (2020a), which was also observed in both subregions of the HF.

5.4.3. Methodological considerations

Two methods were used to quantify the same marker of hippocampal plasticity (DCX) in this thesis: Immunohistochemistry (IHC) in chapters 2 and 3, and gene expression measured using quantitative polymerase chain reaction (qPCR) in chapter 4. Though these methods were not compared directly in any of my studies, similar effects of stressors and dark brooders were found between Chapter 3 and 4 using the different methods. In future studies we may expect both methods to yield similar results, though direct comparison of the methods would be needed to confirm that, as was done in mice by Gualtieri *et al.* (2017). In that study, expression of DCX mRNA in the DG was quantified relative to expression of PROX-1, a marker that is present in all granule cells. Very careful dissection of the DG and selection of the reference gene were required to yield the effect similar to that observed using IHC, suggesting that using gene expression to quantify DCX requires more precision.

Another benefit of using IHC is the ability to distinguish between cell morphologies. DCX is expressed in the cytoplasm, therefore the shape of cell bodies can be visualised, and this may provide valuable information about the age or status of an immature neuron (Balthazart and Ball, 2014). Measuring hippocampal plasticity using IHC was, however, very time consuming because of the need for manual cell counting. Automated cell counting following IHC would make this method much faster, though this was not available during the current studies. Using qPCR as an alternative method is less time consuming, and can allow higher numbers of samples to be analysed in a shorter amount of time, leading to higher statistical power.

5.5. General limitations

Statistical power was low in all three studies, especially in Chapter 3. This is partially due to time consuming methods (e.g. cell counting after IHC) meaning it is not feasible to use higher numbers of birds, and partially due to the availability of suitable subject animals, for example the limited number of available birds which could be considered true controls for Chapter 3.

While Chapter 4 used multiple welfare indicators including behavioural (TI and NOT) and physiological (CORT) measures, Chapters 2 and 3 used hippocampal plasticity as the only welfare measure, and would benefit from additional measurements of affective state using behavioural tests or CORT rather than using hippocampal plasticity alone. In Chapter 2, birds were selected from large flocks therefore recapturing birds for repeated CORT samples may not have been feasible, though behavioural observations could have been conducted at group level. Chapter 3 had some information provided by caecal microbiome analysis in addition to hippocampal plasticity. However, due to lack of information about the direction of causality or true functional information provided by the chosen sequencing methods (16S rRNA sequencing), the limited differences in the composition of the caecal microbiome do not add much meaningful support to the predicted effects of FPD as a stressor. It would be interesting to conduct more powerful studies on the microbiome of birds with FPD and other stressful housing related health conditions, using methods which may provide functional information about any taxa which were differentially abundant between groups.

5.6. Recommendations for future research

As previously mentioned in Chapter 2, six months after brains were sampled for that study, more brains were selected from birds with good and poor body condition. It would be interesting to conduct a similar future study, which may be able to replicate the effect of body condition observed by Armstrong *et al.* (2022) in birds housed in furnished cages and multi-tier aviaries.

After the intriguing results from investigating the effects of dark brooders on hippocampal plasticity in this thesis, it would be interesting to apply similar methods to other housing related conditions which have previously been shown to affect plasticity, to investigate whether dark brooders confer resilience to other causes of stress. The design of dark brooders varies between rearing sites. In order to facilitate designing optimal dark brooders, it would be helpful to know which aspects of dark brooding have the greatest effects on conferring resilience. In a study investigating chicks' preferences for different aspects associated with a mother hen (including heat, colour, shape, etc.), Sherry (1981) found that heat was the only factor tested which affected their preferences. It would be interesting to conduct a study investigating whether heat is the key aspect of maternal care which drives differences in hippocampal plasticity compared to conventionally reared birds.

It is unclear how long dark brooders need to be kept on rearing sites to confer stress resilience. Behavioural studies vary in length of time brooders are present, but I am not aware of any existing studies in which brooders were present for less than the 3 weeks in which dark brooders were available to the birds in Chapter 4. A future study should investigate the duration of dark brooder provision on differences in hippocampal plasticity in adult birds which are exposed to stressors. The age of birds used in this thesis varied between studies, with the oldest birds in Chapter 3 being 70 weeks of age when brains were sampled. This suggests that the effects of dark brooders on stress resilience persist late into the production phase, however the low statistical power in Chapter 3 means these effects are not reliable. A similar study should be repeated to investigate whether the effects of dark brooding that we observed in UCMS birds at 25 weeks of age and FPD birds at 70 weeks of age can be replicated at different ages.

Finally, the mechanisms by which dark brooders confer resilience are still unclear. This study did not begin to investigate the proposed inverted-U shaped relationship between chronic stress and hippocampal plasticity, but calls for future studies to do so in order to contribute to our understanding of an early life intervention which may help us to promote stress resilience in hens.

5.7. General conclusions

In conclusion, differences in environmental complexity between flat-deck and multi-tier housing environments were not great enough to cause detectable differences in hippocampal plasticity in birds which were reared on the same conventional rearing site. This suggests that housing complexity alone does not have significant effects on spatial stimulation or chronic stress, however there was some support for FPD, a common health issue which is exacerbated by housing conditions, as a chronic stressor. FPD and UCMS both had the expected effects on hippocampal plasticity in conventionally reared birds, but dark brooder rearing caused the hippocampal plasticity to increase in response to both these stressors. This suggests that dark brooder rearing may change the brain in early life in a way which confers stress resilience, with benefits persisting late into the production period. The mechanisms of the benefits of dark brooders remain unclear, but it is speculated that the relationship between chronic stress and hippocampal plasticity follows an inverted U shaped curve, and provision of a dark brooder early in life shifts the position of birds on this curve to a point which is beneficial for the development of a healthy stress response. Regardless of the mechanisms by which dark

brooders may confer resilience, the findings of this thesis support previous studies on the welfare benefits of dark brooders and add to the call for their wider implementation on rearing farms.

References

Adhikari, B., Jun, S.-R., Kwon, Y. M., Kiess, A. S. and Adhikari, P. (2020) 'Effects of Housing Types on Cecal Microbiota of Two Different Strains of Laying Hens During the Late Production Phase', *Frontiers in Veterinary Science,* 7.

Alberghina, D., Biondi, V., Passantino, A., Giunta, F. and Panzera, M. (2020) 'Plasma Serotonin in Laying Hens (Gallus gallus domesticus) With and Without Foot pad Dermatitis', *International journal of tryptophan research : IJTR,* 13.

Altman, J. (1962) 'Are new neurons formed in the brains of adult mammals?', *Science,* 135(3509), pp. 1127-8.

Anacker, C., Luna, V. M., Stevens, G. S., Millette, A., Shores, R., Jimenez, J. C., Chen, B. and Hen, R. (2018) 'Hippocampal neurogenesis confers stress resilience by inhibiting the ventral dentate gyrus', *Nature,* 559(7712), pp. 98-102.

Anand, K. S. and Dhikav, V. (2012) 'Hippocampus in health and disease: An overview', *Ann Indian Acad Neurol,* 15(4), pp. 239-46.

Andreani, N. A., Donaldson, C. J. and Goddard, M. (2020) 'A reasonable correlation between cloacal and cecal microbiomes in broiler chickens', *Poultry Science*.

Armstrong, E. A., Richards-Rios, P., Addison, L., Sandilands, V., Guy, J. H., Wigley, P., Boswell, T. and Smulders, T. V. (2022) 'Poor body condition is associated with lower hippocampal plasticity and higher gut methanogen abundance in adult laying hens from two housing systems', *Scientific Reports,* 12(1), pp. 15505.

Armstrong, E. A., Rufener, C., Toscano, M. J., Eastham, J. E., Guy, J. H., Sandilands, V., Boswell, T. and Smulders, T. V. (2020a) 'Keel bone fractures induce a depressive-like state in laying hens', *Scientific Reports,* 10(1).

Armstrong, E. A., Voelkl, B., Voegeli, S., Gebhardt-Henrich, S. G., Guy, J. H., Sandilands, V., Boswell, T., Toscano, M. J. and Smulders, T. V. (2020b) 'Cell Proliferation in the Adult Chicken Hippocampus Correlates With Individual Differences in Time Spent in Outdoor Areas and Tonic Immobility', *Frontiers in Veterinary Science,* 7.

Arya, H., Tamta, K., Kumar, A., Arya, S. and Maurya, R. C. (2024) 'Unpredictable chronic mild stress shows neuronal remodeling in multipolar projection neurons of hippocampal complex in postnatal chicks', *Anatomical Science International*.

Atoji, Y., Saito, S. and Wild, J. M. (2006) 'Fiber connections of the compact division of the posterior pallial amygdala and lateral part of the bed nucleus of the stria terminalis in the pigeon (Columba livia)', *Journal of Comparative Neurology,* 499(2), pp. 161-182.

Atoji, Y., Sarkar, S. and Wild, J. M. (2016) 'Proposed homology of the dorsomedial subdivision and V-shaped layer of the avian hippocampus to Ammon's horn and dentate gyrus, respectively', *Hippocampus,* 26(12), pp. 1608-1617.

Balthazart, J. and Ball, G. F. (2014) 'Doublecortin is a highly valuable endogenous marker of adult neurogenesis in canaries', *Brain, Behavior and Evolution,* 84(1), pp. 1-4.

Bannerman, D. M., Rawlins, J. N. P., McHugh, S. B., Deacon, R. M. J., Yee, B. K., Bast, T., Zhang, W. N., Pothuizen, H. H. J. and Feldon, J. (2004) 'Regional dissociations within the hippocampus—memory and anxiety', *Neuroscience & Biobehavioral Reviews,* 28(3), pp. 273-283.

Bannerman, D. M., Sprengel, R., Sanderson, D. J., McHugh, S. B., Rawlins, J. N., Monyer, H. and Seeburg, P. H. (2014) 'Hippocampal synaptic plasticity, spatial memory and anxiety', *Nat Rev Neurosci,* 15(3), pp. 181-92.

Bari, M. S., Kheravii, S. K., Bajagai, Y. S., Wu, S.-B., Keerqin, C. and Campbell, D. L. M. (2022) 'Cecal Microbiota of Free-Range Hens Varied With Different Rearing Enrichments and Ranging Patterns', *Frontiers in Microbiology,* 12.

Barnea, A., Mishal, A. and Nottebohm, F. (2006) 'Social and spatial changes induce multiple survival regimes for new neurons in two regions of the adult brain: An anatomical representation of time?', *Behavioural Brain Research,* 167(1), pp. 63-74.

Barnea, A. and Nottebohm, F. (1994) 'Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees', *Proc Natl Acad Sci U S A,* 91(23), pp. 11217-21.

Barnes, B. and Glass, K. (2018) 'Modelling low pathogenic avian influenza introduction into the commercial poultry industry', *Mathematical Biosciences,* 300, pp. 115-121.

Bestman, M. and Bikker-Ouwejan, J. (2020) 'Predation in Organic and Free-Range Egg Production', *Animals (Basel),* 10(2).

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., Rosoklija, G. B., Stankov, A., Arango, V., Dwork, A. J., Hen, R. and Mann, J. J. (2018) 'Human Hippocampal Neurogenesis Persists throughout Aging', *Cell Stem Cell,* 22(4), pp. 589-599.e5.

Bonnefous, C., Collin, A., Guilloteau, L. A., Guesdon, V., Filliat, C., Réhault-Godbert, S., Rodenburg, T. B., Tuyttens, F. A. M., Warin, L., Steenfeldt, S., Baldinger, L., Re, M., Ponzio, R., Zuliani, A., Venezia, P., Väre, M., Parrott, P., Walley, K., Niemi, J. K. and Leterrier, C. (2022) 'Welfare issues and potential solutions for laying hens in free range and organic production systems: A review based on literature and interviews', *Frontiers in Veterinary Science,* 9.

Borda-Molina, D., Iffland, H., Schmid, M., Müller, R., Schad, S., Seifert, J., Tetens, J., Bessei, W., Bennewitz, J. and Camarinha-Silva, A. (2021) 'Gut Microbial Composition and Predicted Functions Are Not Associated with Feather Pecking and Antagonistic Behavior in Laying Hens', *Life*, 11(3). DOI: 10.3390/life11030235.

Boseret, G., Ball, G. F. and Balthazart, J. (2007) 'The microtubule-associated protein doublecortin is broadly expressed in the telencephalon of adult canaries', *Journal of Chemical Neuroanatomy,* 33(3), pp. 140-154.

Bouillé, C. and Baylé, J. D. (1973) 'Effects of limbic stimulations or lesions on basal and stress-induced hypothalamic-pituitary-adrenocortical activity in the pigeon', *Neuroendocrinology,* 13(4), pp. 264-77.

Brantsæter, M., Nordgreen, J., Rodenburg, T. B., Tahamtani, F. M., Popova, A. and Janczak, A. M. (2016a) 'Exposure to Increased Environmental Complexity during Rearing Reduces Fearfulness and Increases Use of Three-Dimensional Space in Laying Hens (Gallus gallus domesticus)', *Frontiers in Veterinary Science,* 3.

Brantsæter, M., Tahamtani, F. M., Moe, R. O., Hansen, T. B., Orritt, R., Nicol, C. and Janczak, A. M. (2016b) 'Rearing Laying Hens in Aviaries Reduces Fearfulness following Transfer to Furnished Cages', *Frontiers in Veterinary Science,* 3.

Bright, A. and Johnson, E. A. (2011) 'Smothering in commercial free-range laying hens: a preliminary investigation', *Veterinary Record,* 168(19), pp. 512.

Broom, D. M. (1986) 'Indicators of poor welfare', *British Veterinary Journal,* 142(6), pp. 524-526.

Brown, J. P., Couillard-Després, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L. and Kuhn, H. G. (2003) 'Transient expression of doublecortin during adult neurogenesis', *J Comp Neurol,* 467(1), pp. 1-10.

Campbell, A. M., Johnson, A. M., Persia, M. E. and Jacobs, L. (2022) 'Effects of Housing System on Anxiety, Chronic Stress, Fear, and Immune Function in Bovan Brown Laying Hens', *Animals*, 12(14). DOI: 10.3390/ani12141803.

Campbell, D. L. M., Bari, M. S. and Rault, J. L. (2021) 'Free-range egg production: its implications for hen welfare', *Animal Production Science,* 61(10), pp. 848-855.

Campbell, D. L. M., De Haas, E. N. and Lee, C. (2019) 'A review of environmental enrichment for laying hens during rearing in relation to their behavioral and physiological development', *Poultry Science,* 98(1), pp. 9-28.

Campbell, D. L. M., Dickson, E. J. and Lee, C. (2019) 'Application of open field, tonic immobility, and attention bias tests to hens with different ranging patterns', *PeerJ,* 7, pp. e8122.

Campbell, D. L. M., Talk, A. C., Loh, Z. A., Dyall, T. R. and Lee, C. (2018) 'Spatial Cognition and Range Use in Free-Range Laying Hens', *Animals,* 8(2), pp. 26.

Campbell, S. and Macqueen, G. (2004) 'The role of the hippocampus in the pathophysiology of major depression', *J Psychiatry Neurosci,* 29(6), pp. 417-26.

Campderrich, I., Nazar, F. N., Wichman, A., Marin, R. H., Estevez, I. and Keeling, L. J. (2019) 'Environmental complexity: A buffer against stress in the domestic chick', *PLoS ONE,* 14(1).

Candelotto, L., Stratmann, A., Gebhardt-Henrich, S. G., Rufener, C., van de Braak, T. and Toscano, M. J. (2017) 'Susceptibility to keel bone fractures in laying hens and the role of genetic variation', *Poultry Science,* 96(10), pp. 3517-3528.

Casey-Trott, T., Heerkens, J. L. T., Petrik, M., Regmi, P., Schrader, L., Toscano, M. J. and Widowski, T. (2015) 'Methods for assessment of keel bone damage in poultry', *Poultry Science,* 94(10), pp. 2339-2350.

Champagne, D. L., Bagot, R. C., van Hasselt, F., Ramakers, G., Meaney, M. J., de Kloet, E. R., Joëls, M. and Krugers, H. (2008) 'Maternal care and hippocampal plasticity: evidence for

experience-dependent structural plasticity, altered synaptic functioning, and differential responsiveness to glucocorticoids and stress', *J Neurosci,* 28(23), pp. 6037-45.

Chen, J., Tellez, G. and Escobar, J. (2016) 'Identification of Biomarkers for Footpad Dermatitis Development and Wound Healing', *Frontiers in Cellular and Infection Microbiology,* 6.

Conrad, C. D., Lupien, S. J. and McEwen, B. S. (1999) 'Support for a Bimodal Role for Type II Adrenal Steroid Receptors in Spatial Memory', *Neurobiology of Learning and Memory,* 72(1), pp. 39-46.

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H. G. and Aigner, L. (2005) 'Doublecortin expression levels in adult brain reflect neurogenesis', *Eur J Neurosci,* 21(1), pp. 1-14.

David, D. J., Samuels, B. A., Rainer, Q., Wang, J. W., Marsteller, D., Mendez, I., Drew, M., Craig, D. A., Guiard, B. P., Guilloux, J. P., Artymyshyn, R. P., Gardier, A. M., Gerald, C., Antonijevic, I. A., Leonardo, E. D. and Hen, R. (2009) 'Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression', *Neuron,* 62(4), pp. 479-93.

Davison, T. F., Freeman, B. M. and Rea, J. (1985) 'Effects of continuous treatment with synthetic ACTH1–24 or corticosterone on immature Gallus domesticus', *General and Comparative Endocrinology,* 59(3), pp. 416-423.

De Haas, E. N., Newberry, R. C., Edgar, J., Riber, A. B., Estevez, I., Ferrante, V., Hernandez, C. E., Kjaer, J. B., Ozkan, S., Dimitrov, I., Rodenburg, T. B. and Janczak, A. M. (2021) 'Prenatal and Early Postnatal Behavioural Programming in Laying Hens, With Possible Implications for the Development of Injurious Pecking', *Frontiers in Veterinary Science,* 8.

de Jong, I. C., Schokker, D., Gunnink, H., van Wijhe, M. and Rebel, J. M. J. (2022) 'Early life environment affects behavior, welfare, gut microbiome composition, and diversity in broiler chickens', *Frontiers in Veterinary Science,* 9.

Dickens, M., Romero, L. M., Cyr, N. E., Dunn, I. C. and Meddle, S. L. (2009) 'Chronic stress alters glucocorticoid receptor and mineralocorticoid receptor mRNA expression in the European starling (Sturnus vulgaris) brain', *Journal of Neuroendocrinology,* 21(10), pp. 832-840.

Dimitrov, E. L., Tsuda, M. C., Cameron, H. A. and Usdin, T. B. (2014) 'Anxiety- and depression-like behavior and impaired neurogenesis evoked by peripheral neuropathy persist following resolution of prolonged tactile hypersensitivity', *J Neurosci,* 34(37), pp. 12304-12.

Dranovsky, A. and Hen, R. (2006) 'Hippocampal Neurogenesis: Regulation by Stress and Antidepressants', *Biological Psychiatry,* 59(12), pp. 1136-1143.

Dumontier, L., Janczak, A. M., Smulders, T. V. and Nordgreen, J. (2023) 'Effects of the rearing environment complexity on laying hens' spatial cognition: A holeboard test approach', *Applied Animal Behaviour Science,* 260, pp. 105878.

Duncan, I. J. H. (2001) 'The pros and cons of cages', *World's Poultry Science Journal,* 57(4), pp. 381-390.

Duque, A. and Rakic, P. (2011) 'Different effects of bromodeoxyuridine and [3H]thymidine incorporation into DNA on cell proliferation, position, and fate', *J Neurosci,* 31(42), pp. 15205-17.

Edgar, J., Held, S., Jones, C. and Troisi, C. (2016) 'Influences of Maternal Care on Chicken Welfare', *Animals*, 6(1). DOI: 10.3390/ani6010002.

El-Lethey, H., Aerni, V., Jungi, T. W. and Wechsler, B. (2000) 'Stress and feather pecking in laying hens in relation to housing conditions', *British Poultry Science,* 41(1), pp. 22-28.

Eriksson, P. S., Perfilieva, E., Björk-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998) 'Neurogenesis in the adult human hippocampus', *Nature Medicine,* 4(11), pp. 1313-1317.

Farooq, R. K., Isingrini, E., Tanti, A., Le Guisquet, A.-M., Arlicot, N., Minier, F., Leman, S., Chalon, S., Belzung, C. and Camus, V. (2012) 'Is unpredictable chronic mild stress (UCMS) a reliable model to study depression-induced neuroinflammation?', *Behavioural Brain Research,* 231(1), pp. 130-137.

Felix-Ortiz, Ada C., Beyeler, A., Seo, C., Leppla, Christopher A., Wildes, C. P. and Tye, Kay M. (2013) 'BLA to vHPC Inputs Modulate Anxiety-Related Behaviors', *Neuron,* 79(4), pp. 658-664.

Ferchmin, P. A. and Eterović, V. A. (1977) 'Brain plasticity and environmental complexity: Role of motor skills', *Physiology and Behavior,* 18(3), pp. 455-461.

Fiala, B. A., Joyce, J. N. and Greenough, W. T. (1978) 'Environmental complexity modulates growth of granule cell dendrites in developing but not adult hippocampus of rats', *Experimental Neurology,* 59(3), pp. 372-383.

Forkman, B., Boissy, A., Meunier-Salaün, M. C., Canali, E. and Jones, R. B. (2007) 'A critical review of fear tests used on cattle, pigs, sheep, poultry and horses', *Physiology & Behavior,* 92(3), pp. 340-374.

Forslind, S., Hernandez, C. E., Riber, A. B., Wall, H. and Blokhuis, H. J. (2022) 'Resting behavior of broilers reared with or without artificial brooders', *Frontiers in Veterinary Science,* 9.

Foster, J. A., Rinaman, L. and Cryan, J. F. (2017) 'Stress & the gut-brain axis: Regulation by the microbiome', *Neurobiology of Stress,* 7, pp. 124-136.

Fraisse, F. and Cockrem, J. F. (2006) 'Corticosterone and fear behaviour in white and brown caged laying hens', *British Poultry Science,* 47(2), pp. 110-119.

Gallup, G. G. and Suarez, S. D. (1980) 'An ethological analysis of open-field behaviour in chickens', *Animal Behaviour,* 28(2), pp. 368-378.

Gebhardt-Henrich, S. G. and Fröhlich, E. K. F. (2015) 'Early Onset of Laying and Bumblefoot Favor Keel Bone Fractures', *Animals*, 5(4), pp. 1192-1206. DOI: 10.3390/ani5040406.

Gebhardt-Henrich, S. G., Toscano, M. J. and Fröhlich, E. K. F. (2014) 'Use of outdoor ranges by laying hens in different sized flocks', *Applied Animal Behaviour Science,* 155, pp. 74-81.

Giersberg, M. F., Molenaar, R., de Jong, I. C., Souza da Silva, C., van den Brand, H., Kemp, B. and Rodenburg, T. B. (2021) 'Effects of hatching system on the welfare of broiler chickens in early and later life', *Poultry Science,* 100(3), pp. 100946.

Gilani, A.-M., Knowles, T. G. and Nicol, C. J. (2012) 'The effect of dark brooders on feather pecking on commercial farms', *Applied Animal Behaviour Science,* 142(1), pp. 42-50.

Grafen, A. (1988) 'On the uses of data on lifetime reproductive success', in Clutton-Brock, T.H. (ed.) *Reproductive success*: University of Chicago Press, pp. 454-71.

Gray, H., Davies, R., Bright, A., Rayner, A. and Asher, L. (2020) 'Why Do Hens Pile? Hypothesizing the Causes and Consequences', *Frontiers in Veterinary Science,* 7.

Grégoire, C. A., Bonenfant, D., Le Nguyen, A., Aumont, A. and Fernandes, K. J. L. (2014) 'Untangling the influences of voluntary running, environmental complexity, social housing and stress on adult hippocampal neurogenesis', *PLoS ONE,* 9(1).

Gualtieri, F., Armstrong, E. A., Longmoor, G. K., D'Eath, R. B., Sandilands, V., Boswell, T. and Smulders, T. V. (2019) 'Unpredictable Chronic Mild Stress Suppresses the Incorporation of

New Neurons at the Caudal Pole of the Chicken Hippocampal Formation', *Scientific Reports,* 9(1).

Gualtieri, F., Brégère, C., Laws, G. C., Armstrong, E. A., Wylie, N. J., Moxham, T. T., Guzman, R., Boswell, T. and Smulders, T. V. (2017) 'Effects of Environmental Enrichment on Doublecortin and BDNF Expression along the Dorso-Ventral Axis of the Dentate Gyrus', *Frontiers in Neuroscience,* 11(488).

Gunnarsson, S., Matthews, L. R., Foster, T. M. and Temple, W. (2000) 'The demand for straw and feathers as litter substrates by laying hens', *Applied Animal Behaviour Science,* 65(4), pp. 321-330.

Gupta, S., Maurya, R., Saxena, M. and Sen, J. (2012) 'Defining structural homology between the mammalian and avian hippocampus through conserved gene expression patterns observed in the chick embryo', *Dev Biol,* 366(2), pp. 125-41.

Habig, C. and Distl, O. (2013) 'Evaluation of bone strength, keel bone status, plumage condition and egg quality of two layer lines kept in small group housing systems', *Br Poult Sci,* 54(4), pp. 413-24.

Hajjo, H. and Geva-Zatorsky, N. (2019) 'Gut microbiota - host interactions now also brain-immune axis', *Curr Opin Neurobiol,* 62, pp. 53-59.

Harlander-Matauschek, A., Rodenburg, T. B., Sandilands, V., Tobalske, B. W. and Toscano, M. J. (2015) 'Causes of keel bone damage and their solutions in laying hens', *World's Poultry Science Journal,* 71(3), pp. 461-472.

Heerkens, J. L. T. (2016) *Lifting laying hen welfare to a higher level.* Doctor of Veterinary Sciences, Ghent University, Merelbeke, Belgium.

Heidemann Olsen, R., Christensen, H., Kabell, S. and Bisgaard, M. (2018) 'Characterization of prevalent bacterial pathogens associated with pododermatitis in table egg layers', *Avian Pathology,* 47(3), pp. 281-285.

Herold, C., Bingman, V. P., Ströckens, F., Letzner, S., Sauvage, M., Palomero-Gallagher, N., Zilles, K. and Güntürkün, O. (2014) 'Distribution of neurotransmitter receptors and zinc in the pigeon (Columba livia) hippocampal formation: A basis for further comparison with the mammalian hippocampus', *Journal of Comparative Neurology,* 522(11), pp. 2553-2575.

Hester, P. Y. (1994) 'The Role of Environment and Management on Leg Abnormalities in Meat-Type Fowl1', *Poultry Science,* 73(6), pp. 904-915.

Hester, P. Y., Enneking, S. A., Haley, B. K., Cheng, H. W., Einstein, M. E. and Rubin, D. A. (2013) 'The effect of perch availability during pullet rearing and egg laying on musculoskeletal health of caged White Leghorn hens', *Poultry Science,* 92(8), pp. 1972-1980.

Hill, M. N., Hellemans, K. G., Verma, P., Gorzalka, B. B. and Weinberg, J. (2012) 'Neurobiology of chronic mild stress: parallels to major depression', *Neurosci Biobehav Rev,* 36(9), pp. 2085-117.

Hofer, M., Pagliusi, S. R., Hohn, A., Leibrock, J. and Barde, Y. A. (1990) 'Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain', *The EMBO Journal,* 9(8), pp. 2459-2464-2464.

Holick, K. A., Lee, D. C., Hen, R. and Dulawa, S. C. (2008) 'Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor', *Neuropsychopharmacology,* 33(2), pp. 406-17.

Hothersall, B., Caplen, G., Parker, R., Nicol, C., Waterman-Pearson, A., Weeks, C. and Murrell, J. (2016) 'Effects of carprofen, meloxicam and butorphanol on broiler chickens' performance in mobility tests', *Animal Welfare,* 25, pp. 55-67.

Hu, V. W., Black, G. E., Torres-Duarte, A. and Abramson, F. P. (2002) '3H-thymidine is a defective tool with which to measure rates of DNA synthesis', *Faseb j,* 16(11), pp. 1456-7.

Ide, Y., Fujiyama, F., Okamoto-Furuta, K., Tamamaki, N., Kaneko, T. and Hisatsune, T. (2008) 'Rapid integration of young newborn dentate gyrus granule cells in the adult hippocampal circuitry', *Eur J Neurosci,* 28(12), pp. 2381-92.

Jacobson, L. and Sapolsky, R. (1991) 'The Role of the Hippocampus in Feedback Regulation of the Hypothalamic-Pituitary-Adrenocortical Axis*', *Endocrine Reviews,* 12(2), pp. 118-134.

Janczak, A. M. and Riber, A. B. (2015) 'Review of rearing-related factors affecting the welfare of laying hens', *Poultry Science,* 94(7), pp. 1454-1469.

Janda, J. M. and Abbott, S. L. (2007) '16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls', *J Clin Microbiol,* 45(9), pp. 2761-4.

Jansen, S., Bues, M., Baulain, U., Habig, C., Halle, I., Petow, S., Sharifi, A. R., Weigend, A., Wilkens, M. R. and Weigend, S. (2020) 'Bone Health or Performance? Adaptation Response of Genetically Divergent Chicken Layer Lines to a Nutritive Calcium Depletion', *Animals (Basel),* 10(9).

Jensen, A. B., Palme, R. and Forkman, B. (2006) 'Effect of brooders on feather pecking and cannibalism in domestic fowl (Gallus gallus domesticus)', *Applied Animal Behaviour Science,* 99(3), pp. 287-300.

Jones, R. B. (1992) 'The nature of handling immediately prior to test affects tonic immobility fear reactions in laying hens and broilers', *Applied Animal Behaviour Science,* 34(3), pp. 247-254.

Jones, R. B. (1996) 'Fear and adaptability in poultry: insights, implications and imperatives', *World's Poultry Science Journal,* 52(2), pp. 131-174.

Jones, R. B. and Waddington, D. (1992) 'Modification of fear in domestic chicks, Gallus gallus domesticus, via regular handling and early environmental enrichment', *Animal Behaviour,* 43(6), pp. 1021-1033.

Kellner, Y., Goedecke, N., Dierkes, T., Thieme, N., Zagrebelsky, M. and Korte, M. (2014) 'The BDNF effects on dendritic spines of mature hippocampal neurons depend on neuronal activity', *Frontiers in Synaptic Neuroscience,* 6.

Kelman, Z. (1997) 'PCNA: structure, functions and interactions', *Oncogene,* 14(6), pp. 629-640.

Kheirbek, M. A., Drew, L. J., Burghardt, N. S., Costantini, D. O., Tannenholz, L., Ahmari, S. E., Zeng, H., Fenton, A. A. and Hen, R. (2013) 'Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus', *Neuron,* 77(5), pp. 955-68.

Kirby, E. D., Muroy, S. E., Sun, W. G., Covarrubias, D., Leong, M. J., Barchas, L. A. and Kaufer, D. (2013) 'Acute stress enhances adult rat hippocampal neurogenesis and activation of newborn neurons via secreted astrocytic FGF2', *eLife,* 2, pp. e00362.

Knierim, J. J. (2015) 'The hippocampus', *Current Biology,* 25(23), pp. R1116-R1121.

Kremer, T., Jagasia, R., Herrmann, A., Matile, H., Borroni, E., Francis, F., Kuhn, H. G. and Czech, C. (2013) 'Analysis of Adult Neurogenesis: Evidence for a Prominent "Non-Neurogenic" DCX-Protein Pool in Rodent Brain', *PLoS ONE,* 8(5).

La Rosa, C., Cavallo, F., Pecora, A., Chincarini, M., Ala, U., Faulkes, C. G., Nacher, J., Cozzi, B., Sherwood, C. C., Amrein, I. and Bonfanti, L. (2020) 'Phylogenetic variation in cortical layer II immature neuron reservoir of mammals', *eLife,* 9, pp. e55456.

Lay, D. C., Jr., Fulton, R. M., Hester, P. Y., Karcher, D. M., Kjaer, J. B., Mench, J. A., Mullens, B. A., Newberry, R. C., Nicol, C. J., O'Sullivan, N. P. and Porter, R. E. (2011) 'Hen welfare in different housing systems', *Poult Sci,* 90(1), pp. 278-94.

Lecomte, V., Kaakoush, N. O., Maloney, C. A., Raipuria, M., Huinao, K. D., Mitchell, H. M. and Morris, M. J. (2015) 'Changes in Gut Microbiota in Rats Fed a High Fat Diet Correlate with Obesity-Associated Metabolic Parameters', *PLOS ONE,* 10(5), pp. e0126931.

Lenth, R. V. (2024) *emmeans: Estimated Marginal Means, aka Least-Squares Means*. R package version 1.10.0. Available at: https://CRAN.R-project.org/package=emmeans.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P. M. and Meaney, M. J. (1997) 'Maternal care, hippocampal glucocorticoid receptors, and hypothalamic- pituitary-adrenal responses to stress', *Science,* 277(5332), pp. 1659-1662.

Lourenço-Silva, M. I., Ulans, A., Campbell, A. M., Almeida Paz, I. C. L. and Jacobs, L. (2023) 'Social-pair judgment bias testing in slow-growing broiler chickens raised in low- or high-complexity environments', *Scientific Reports,* 13(1).

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology,* 15(12), pp. 550.

MacLachlan, S. S., Ali, A. B. A., Toscano, M. J. and Siegford, J. M. (2020) 'Influence of later exposure to perches and nests on flock level distribution of hens in an aviary system during lay', *Poultry Science,* 99(1), pp. 30-38.

Marasco, V., Herzyk, P., Robinson, J. and Spencer, K. A. (2016) 'Pre- and Post-Natal Stress Programming: Developmental Exposure to Glucocorticoids Causes Long-Term Brain-Region Specific Changes to Transcriptome in the Precocial Japanese Quail', *Journal of Neuroendocrinology,* 28(5).

Marin, R. H., Freytes, P., Guzman, D. and Bryan Jones, R. (2001) 'Effects of an acute stressor on fear and on the social reinstatement responses of domestic chicks to cagemates and strangers', *Applied Animal Behaviour Science,* 71(1), pp. 57-66.

Matur, E., Eraslan, E., Akyazi, I., Ergul Ekiz, E., Eseceli, H., Keten, M., Metiner, K. and Aktaran Bala, D. (2015) 'The effect of furnished cages on the immune response of laying hens under social stress', *Poultry Science,* 94(12), pp. 2853-2862.

McMurdie, P. J. and Holmes, S. (2013) 'phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data', *PLOS ONE,* 8(4), pp. e61217.

Miranda, M., Morici, J. F., Zanoni, M. B. and Bekinschtein, P. (2019) 'Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain', *Frontiers in Cellular Neuroscience,* 13.

Montalcini, C. M., Petelle, M. B. and Toscano, M. J. (2023) 'Commercial hatchery practices have long-lasting effects on laying hens' spatial behaviour and health', *PLOS ONE,* 18(12), pp. e0295560.

Moser, M. B. and Moser, E. I. (1998) 'Functional differentiation in the hippocampus', *Hippocampus,* 8(6), pp. 608-619.

Nasr, M. A. F., Murrell, J., Wilkins, L. J. and Nicol, C. J. (2012) 'The effect of keel fractures on egg-production parameters, mobility and behaviour in individual laying hens', *Animal Welfare,* 21(1), pp. 127-135.

Nasr, M. A. F., Nicol, C. J. and Murrell, J. C. (2012) 'Do Laying Hens with Keel Bone Fractures Experience Pain?', *PLOS ONE,* 7(8), pp. e42420.

Nguyen, H. B., Bagot, R. C., Diorio, J., Wong, T. P. and Meaney, M. J. (2015) 'Maternal care differentially affects neuronal excitability and synaptic plasticity in the dorsal and ventral hippocampus', *Neuropsychopharmacology,* 40(7), pp. 1590-1599.

Nguyen, H. B., Parent, C., Tse, Y. C., Wong, T. P. and Meaney, M. J. (2018) 'Generalization of Conditioned Auditory Fear is Regulated by Maternal Effects on Ventral Hippocampal Synaptic Plasticity', *Neuropsychopharmacology,* 43(6), pp. 1297-1307.

Nollet, M. (2021) 'Models of Depression: Unpredictable Chronic Mild Stress in Mice', *Current Protocols,* 1(8), pp. e208.

Nordquist, R. E., Heerkens, J. L. T., Rodenburg, T. B., Boks, S., Ellen, E. D. and van der Staay, F. J. (2011) 'Laying hens selected for low mortality: Behaviour in tests of fearfulness, anxiety and cognition', *Applied Animal Behaviour Science,* 131(3), pp. 110-122.

Nordquist, R. E., Zeinstra, E. C., Dougherty, A. and Riber, A. B. (2020) 'Effects of Dark Brooder Rearing and Age on Hypothalamic Vasotocin and Feather Corticosterone Levels in Laying Hens', *Frontiers in Veterinary Science,* 7(19).

Nordquist, R. E., Zeinstra, E. C., Rodenburg, T. B. and van der Staay, F. J. (2013) 'Effects of maternal care and selection for low mortality on tyrosine hydroxylase concentrations and cell soma size in hippocampus and nidopallium caudolaterale in adult laying hen1', *Journal of Animal Science,* 91(1), pp. 137-146.

Ogbonnaya, E. S., Clarke, G., Shanahan, F., Dinan, T. G., Cryan, J. F. and O'Leary, O. F. (2015) 'Adult Hippocampal Neurogenesis Is Regulated by the Microbiome', *Biological Psychiatry,* 78(4), pp. e7-e9.

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M. N., Lahti, L., McGlinn, D., Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C. and Weedon, J. (2022) *vegan: Community Ecology Package*. R package version 2.6-4. Available at: https://CRAN.R-project.org/package=vegan.

Pagazaurtundua, A. and Warriss, P. D. (2006) 'Levels of foot pad dermatitis in broiler chickens reared in 5 different systems', *Br Poult Sci,* 47(5), pp. 529-32.

Payne, H. L., Lynch, G. F. and Aronov, D. (2021) 'Neural representations of space in the hippocampus of a food-caching bird', *Science (New York, N.Y.),* 373(6552), pp. 343-348.

Pettersson, I. C., Freire, R. and Nicol, C. J. (2016) 'Factors affecting ranging behaviour in commercial free-range hens', *World's Poultry Science Journal,* 72(1), pp. 137-150.

Pires, P. G. S., Oliveira, G. S., McManus, C., Santos, V. M. and Moraes, P. O. (2024) 'Impact of housing system on intestinal microbiota of laying hens – A systematic review', *Research in Veterinary Science*, pp. 105184.

Piumatti, M., Palazzo, O., Rosa, C. L., Crociara, P., Parolisi, R., Luzzati, F., Lévy, F. and Bonfanti, L. (2018) 'Non-Newly Generated, "Immature" Neurons in the Sheep Brain Are Not Restricted to Cerebral Cortex', *The Journal of Neuroscience,* 38(4), pp. 826-842.

Poirier, C., Bateson, M., Gualtieri, F., Armstrong, E. A., Laws, G. C., Boswell, T. and Smulders, T. V. (2019) 'Validation of hippocampal biomarkers of cumulative affective experience', *Neuroscience & Biobehavioral Reviews,* 101, pp. 113-121.

Pravosudov, V. V. and Omanska, A. (2005) 'Prolonged moderate elevation of corticosterone does not affect hippocampal anatomy or cell proliferation rates in mountain chickadees (Poecile gambeli)', *Journal of Neurobiology,* 62(1), pp. 82-91.

Pullin, A. N., Farrar, V. S., Loxterkamp, J. W., Jones, C. T., Calisi, R. M., Horback, K., Lein, P. J. and Makagon, M. M. (2022) 'Providing height to pullets does not influence hippocampal dendritic morphology or brain-derived neurotrophic factor at the end of the rearing period', *Poultry Science*, pp. 102161.

Pullin, A. N., Rufener, C. B., Millman, S. T., Tarlton, J. F., Toscano, M. J., Blatchford, R. A. and Makagon, M. M. (2024) 'Providing elevated structures in the pullet rearing environment affects behavior during initial acclimation to a layer aviary', *Poultry Science,* 103(3), pp. 103357.

Pusch, E. A., Bentz, A. B., Becker, D. J. and Navara, K. J. (2018) 'Behavioral phenotype predicts physiological responses to chronic stress in proactive and reactive birds', *General and Comparative Endocrinology,* 255, pp. 71-77.

Puvadolpirod, S. and Thaxton, J. P. (2000) 'Model of physiological stress in chickens 1. Response parameters12', *Poultry Science,* 79(3), pp. 363-369.

R Core Team (2023) *R: A Language and Environment for Statistical Computing*: R Foundation for Statistical Computing. Available at: https://www.R-project.org/.

Regmi, P., Smith, N., Nelson, N., Haut, R. C., Orth, M. W. and Karcher, D. M. (2016) 'Housing conditions alter properties of the tibia and humerus during the laying phase in Lohmann white Leghorn hens1', *Poultry Science,* 95(1), pp. 198-206.

Riber, A. B., Casey-Trott, T. M. and Herskin, M. S. (2018) 'The Influence of Keel Bone Damage on Welfare of Laying Hens', *Frontiers in Veterinary Science,* 5.

Riber, A. B. and Guzman, D. A. (2016) 'Effects of dark brooders on behavior and fearfulness in layers', *Animals,* 6(1).

Riber, A. B. and Guzman, D. A. (2017) 'Effects of different types of dark brooders on injurious pecking damage and production-related traits at rear and lay in layers', *Poultry Science,* 96(10), pp. 3529-3538.

Riber, A. B. and Hinrichsen, L. K. (2016) 'Keel-bone damage and foot injuries in commercial laying hens in Denmark', *Animal Welfare,* 25(2), pp. 179-184.

Richards-Rios, P., Fothergill, J., Bernardeau, M. and Wigley, P. (2020a) 'Development of the Ileal Microbiota in Three Broiler Breeds', *Frontiers in Veterinary Science,* 7(17).

Richards-Rios, P., Leeming, G., Fothergill, J., Bernardeau, M. and Wigley, P. (2020b) 'Topical Application of Adult Cecal Contents to Eggs Transplants Spore-Forming Microbiota but Not Other Members of the Microbiota to Chicks', *Applied and Environmental Microbiology,* 86(5), pp. e02387-19.

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C. and Gasbarrini, A. (2017) 'Proteobacteria: A Common Factor in Human Diseases', *Biomed Res Int,* 2017, pp. 9351507.

Robertson, B.-A., Rathbone, L., Cirillo, G., D'Eath, R. B., Bateson, M., Boswell, T., Wilson, P. W., Dunn, I. C. and Smulders, T. V. (2017) 'Food restriction reduces neurogenesis in the avian hippocampal formation', *PloS one,* 12(12), pp. e0189158-e0189158.

Rodenburg, B., Van Krimpen, M., Jong, I., De Haas, E., Kops, M. S., Riedstra, B. J., Nordquist, R., Wagenaar, J. P., Bestman, M. W. P. and Nicol, C. (2013) 'The prevention and control of feather pecking in laying hens: Identifying the underlying principles', *World's Poultry Science Journal,* 69.

Ross, M., Garland, A., Harlander-Matauschek, A., Kitchenham, L. and Mason, G. (2019) 'Welfare-improving enrichments greatly reduce hens' startle responses, despite little change in judgment bias', *Scientific Reports,* 9(1), pp. 11881.

Ross, M., Rausch, Q., Vandenberg, B. and Mason, G. (2020) 'Hens with benefits: Can environmental enrichment make chickens more resilient to stress?', *Physiology & Behavior,* 226, pp. 113077.

Rychlik, I. (2020) 'Composition and Function of Chicken Gut Microbiota', *Animals (Basel),* 10(1).

Samuels, B. A. and Hen, R. (2011) 'Neurogenesis and affective disorders', *Eur J Neurosci,* 33(6), pp. 1152-9.

Sandilands, V., Moinard, C. and Sparks, N. H. (2009) 'Providing laying hens with perches: fulfilling behavioural needs but causing injury?', *British Poultry Science,* 50(4), pp. 395-406.

Schloss, P. D. and Westcott, S. L. (2011) 'Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis', *Appl Environ Microbiol,* 77(10), pp. 3219-26.

Scoville, W. B. and Milner, B. (1957) 'Loss of recent memory after bilateral hippocampal lesions', *Journal of neurology, neurosurgery, and psychiatry,* 20(1), pp. 11-21.

Sheng, J. A., Bales, N. J., Myers, S. A., Bautista, A. I., Roueinfar, M., Hale, T. M. and Handa, R. J. (2021) 'The Hypothalamic-Pituitary-Adrenal Axis: Development, Programming Actions of Hormones, and Maternal-Fetal Interactions', *Frontiers in Behavioral Neuroscience,* 14.

Shepherd, E. M. and Fairchild, B. D. (2010) 'Footpad dermatitis in poultry', *Poultry Science,* 89(10), pp. 2043-2051.

Sherry, D. F. (1981) 'Parental Care and the Development of Thermoregulation in Red Junglefowl', *Behaviour,* 76(3-4), pp. 250-279.

Shi, S., Qi, Z., Gu, B., Cheng, B., Tu, J., Song, X., Shao, Y., Liu, H., Qi, K. and Li, S. (2019) 'Analysis of high-throughput sequencing for cecal microbiota diversity and function in hens under different rearing systems', *3 Biotech,* 9(12).

Sinclair, M., Lee, N. Y. P., Hötzel, M. J., de Luna, M. C. T., Sharma, A., Idris, M., Islam, M. A., Iyasere, O. S., Navarro, G., Ahmed, A. A., Curry, M., Burns, G. L. and Marchant, J. N. (2022) 'Consumer attitudes towards egg production systems and hen welfare across the world', *Frontiers in Animal Science,* 3.

Singmann, H., Bolker, B., Westfall, J., Aust, F. and Ben-Shachar, M. (2023) *afex: Analysis of Factorial Experiments*. R package version 1.3-0. Available at: https://CRAN.R-project.org/package=afex.

Sirovnik, J. and Riber, A. B. (2022) 'Why-Oh-Why? Dark Brooders Reduce Injurious Pecking, Though Are Still Not Widely Used in Commercial Rearing of Layer Pullets', *Animals*, 12(10). DOI: 10.3390/ani12101276.

Skånberg, L., Newberry, R. C., Estevez, I. and Keeling, L. J. (2023) 'Environmental change or choice during early rearing improves behavioural adaptability in laying hen chicks', *Scientific Reports,* 13(1), pp. 6178.

Smail, M. A., Smith, B. L., Nawreen, N. and Herman, J. P. (2020) 'Differential impact of stress and environmental enrichment on corticolimbic circuits', *Pharmacology Biochemistry and Behavior,* 197, pp. 172993.

Smulders, T. V. (2017) 'The Avian Hippocampal Formation and the Stress Response', *Brain Behav Evol,* 90(1), pp. 81-91.

Smulders, T. V. (2021) 'Telencephalic regulation of the HPA axis in birds', *Neurobiology of Stress,* 15, pp. 100351.

Sorrells, S. F., Paredes, M. F., Cebrian-Silla, A., Sandoval, K., Qi, D., Kelley, K. W., James, D., Mayer, S., Chang, J., Auguste, K. I., Chang, E. F., Gutierrez, A. J., Kriegstein, A. R., Mathern, G. W., Oldham, M. C., Huang, E. J., Garcia-Verdugo, J. M., Yang, Z. and Alvarez-Buylla, A. (2018) 'Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults', *Nature,* 555(7696), pp. 377-381.

Stadig, L. M., Ampe, B. A., Van Gansbeke, S., Van den Bogaert, T., D'Haenens, E., Heerkens, J. L. T. and Tuyttens, F. A. M. (2016) 'Survey of egg farmers regarding the ban on conventional cages in the EU and their opinion of alternative layer housing systems in Flanders, Belgium', *Poultry Science,* 95(3), pp. 715-725.

Stanley, D., Geier, M. S., Chen, H., Hughes, R. J. and Moore, R. J. (2015) 'Comparison of fecal and cecal microbiotas reveals qualitative similarities but quantitative differences', *BMC Microbiology,* 15(1).

Suarez, S. D. and Gallup, G. G. (1983) 'Social reinstatement and open-field testing in chickens', *Animal Learning & Behavior,* 11(1), pp. 119-126.

Sun, B., Hou, L. and Yang, Y. (2021) 'The Development of the Gut Microbiota and Short-Chain Fatty Acids of Layer Chickens in Different Growth Periods', *Front Vet Sci,* 8, pp. 666535.

Tamta, K., Kumar, A., Arya, H., Arya, S. and Maurya, Ram C. (2023) 'Neuronal plasticity in hippocampal neurons due to chronic mild stress and after stress removal in postnatal chicks', *Journal of Anatomy,* 00(n/a).

Terada, M., Kuzumaki, N., Hareyama, N., Imai, S., Niikura, K., Narita, M., Yamazaki, M., Suzuki, T. and Narita, M. (2008) 'Suppression of enriched environment-induced neurogenesis in a rodent model of neuropathic pain', *Neuroscience Letters,* 440(3), pp. 314-318.

Thernau, T. (2023) *A Package for Survival Analysis in R*. R package version 3.5-5. Available at: https://CRAN.R-project.org/package=survival.

Thernau, T. M. (2022) *coxme: Mixed Effects Cox Models*. R package version 2.2-18.1. Available at: https://CRAN.R-project.org/package=coxme.

Thøfner, I., Hougen, H. P., Villa, C., Lynnerup, N. and Christensen, J. P. (2020) 'Pathological characterization of keel bone fractures in laying hens does not support external trauma as the underlying cause', *PLOS ONE,* 15(3), pp. e0229735.

Tilocca, B., Witzig, M., Rodehutscord, M. and Seifert, J. (2016) 'Variations of Phosphorous Accessibility Causing Changes in Microbiome Functions in the Gastrointestinal Tract of Chickens', *PLOS ONE,* 11(10), pp. e0164735.

van der Eijk, J. A. J., de Vries, H., Kjaer, J. B., Naguib, M., Kemp, B., Smidt, H., Rodenburg, T. B. and Lammers, A. (2019) 'Differences in gut microbiota composition of laying hen lines divergently selected on feather pecking', *Poult Sci,* 98(12), pp. 7009-7021.

van der Eijk, J. A. J., Rodenburg, T. B., de Vries, H., Kjaer, J. B., Smidt, H., Naguib, M., Kemp, B. and Lammers, A. (2020) 'Early-life microbiota transplantation affects behavioural responses, serotonin and immune characteristics in chicken lines divergently selected on feather pecking', *Scientific Reports,* 10(1).

van Niekerk, T. G. C. M., Gunnink, H. and van Reenen, C. G. (2012) *Welfare Quality assessment protocol for laying hens = Welfare Quality assessment protocol voor leghennen*, Lelystad: Wageningen UR Livestock Research (589. Available at: https://edepot.wur.nl/235525.

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D. and Gage, F. H. (2002) 'Functional neurogenesis in the adult hippocampus', *Nature,* 415(6875), pp. 1030-4.

Varriale, L., Coretti, L., Dipineto, L., Green, B. D., Pace, A., Lembo, F., Menna, L. F., Fioretti, A. and Borrelli, L. (2022) 'An Outdoor Access Period Improves Chicken Cecal Microbiota and Potentially Increases Micronutrient Biosynthesis', *Frontiers in Veterinary Science,* 9.

Viana da Silva, S., Zhang, P., Haberl, M. G., Labrousse, V., Grosjean, N., Blanchet, C., Frick, A. and Mulle, C. (2019) 'Hippocampal Mossy Fibers Synapses in CA3 Pyramidal Cells Are Altered at an Early Stage in a Mouse Model of Alzheimer's Disease', *J Neurosci,* 39(21), pp. 4193-4205.

Walker, D. J., Zimmer, C., Larriva, M., Healy, S. D. and Spencer, K. A. (2019) 'Early-life adversity programs long-term cytokine and microglia expression within the HPA axis in female Japanese quail', *Journal of Experimental Biology,* 222(6).

Walsh, R. N., Budtz-Olsen, O. E., Penny, J. E. and Cummins, R. A. (1969) 'The effects of environmental complexity on the histology of the rat hippocampus', *Journal of Comparative Neurology,* 137(3), pp. 361-365.

Wang, G., Ekstrand, C. and Svedberg, J. (1998) 'Wet litter and perches as risk factors for the development of foot pad dermatitis in floor-housed hens', *Br Poult Sci,* 39(2), pp. 191-7.

Wei, H., Feng, Y., Ding, S., Nian, H., Yu, H., Zhao, Q., Bao, J. and Zhang, R. (2022) 'Keel bone damage affects behavioral and physiological responses related to stress and fear in two strains of laying hens', *Journal of Animal Science,* 100(4).

Weitzenbürger, D., Vits, A., Hamann, H., Hewicker-Trautwein, M. and Distl, O. (2006) 'Macroscopic and histopathological alterations of foot pads of laying hens kept in small group housing systems and furnished cages', *British Poultry Science,* 47(5), pp. 533-543.

Wierup, M., Wahlström, H., Lahti, E., Eriksson, H., Jansson, D. S., Odelros, Å. and Ernholm, L. (2017) 'Occurrence of Salmonella spp.: a comparison between indoor and outdoor housing of broilers and laying hens', *Acta Veterinaria Scandinavica,* 59(1), pp. 13.

Willner, P., Towell, A., Sampson, D., Sophokleous, S. and Muscat, R. (1987) 'Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant', *Psychopharmacology,* 93(3), pp. 358-364.

Wood, D. E., Lu, J. and Langmead, B. (2019) 'Improved metagenomic analysis with Kraken 2', *Genome Biology,* 20(1), pp. 257.

Yang, T., Nie, Z., Shu, H., Kuang, Y., Chen, X., Cheng, J., Yu, S. and Liu, H. (2020) 'The Role of BDNF on Neural Plasticity in Depression', *Frontiers in Cellular Neuroscience,* 14.

Zhang, J. and Jiao, J. (2015) 'Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis', *BioMed Research International,* 2015, pp. 727542.

Zidar, J., Campderrich, I., Jansson, E., Wichman, A., Winberg, S., Keeling, L. and Løvlie, H. (2018) 'Environmental complexity buffers against stress-induced negative judgement bias in female chickens', *Scientific Reports,* 8(1), pp. 5404.

Zimmerman, P. H., Buijs, S. A. F., Bolhuis, J. E. and Keeling, L. J. (2011) 'Behaviour of domestic fowl in anticipation of positive and negative stimuli', *Animal Behaviour,* 81(3), pp. 569-577.