Adult Hippocampal Neurogenesis as a Biomarker of Long-Term Experience in Domestic Laying Hens (*Gallus gallus domesticus*)

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Abstract

Consideration for the welfare of farmed animals has highlighted the goal of ensuring that they lead a “life worth living”, comprised of an acceptable balance between positive and negative experiences. Novel objective biomarkers of long-term or cumulative experience that are valence-specific may contribute to the assessment of this criterion. In humans, numbers of surviving hippocampal neurons produced through adult neurogenesis reflect self-reported mood. In rats and mice, adult hippocampal neurogenesis (AHN) is reliably suppressed by chronic stress, but stimulated by positive experiences. As AHN in the avian brain also appears to be downregulated by chronic stress and upregulated by cognitive enrichment, this project sought to quantify immature neurons in the brains of laying hens with known long-term experiences, to identify factors likely to have a notable influence on lifetime subjective welfare. Four studies measured levels of AHN in hens in relation to *i)* experimental housing conditions, *ii)* individual differences in use of outdoor areas, *iii)* severity of acquired fractures to the keel bone, and *iv)* alternative commercial housing systems (enriched-cage versus aviary) and physical body condition, on-farm. Over the course of the project, a methodological comparison was made between traditional morphological quantification of immature neurons by immunohistochemistry, and quantification of the transcription of genetic markers of AHN. Densities of immature differentiating neurons were suppressed by severe keel bone fractures and in association with poor feather coverage, low body mass and pale combs. The proliferative stage of AHN was also positively associated with the time that individual hens within a free-range system spent in the outdoor areas provided. The results suggest that interventions to reduce the prevalence of keel bone fractures and improve physical condition in commercial laying hens are likely to have significant benefits for the balance of cumulative chronic stress and positive affect experienced.

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The research project detailed in Chapter 5 was conducted in collaboration with industrial partners at a local egg production farm in the northeast of England and a pullet rearing farm in Shropshire. No experimental manipulation was conducted for this study, and animals were managed by their respective production companies until the day of sampling. Focal hens were selected in conjunction with the farm's production managers and transported to Newcastle University for tissue collection. Caecal samples were sent to the University of Liverpool for analysis of composition of the microbiome, which was conducted by collaborator Peter Richards (supervised by Paul Wigley). Peter also carried out the Gneiss analyses of the microbiome data reported in section 5.4.4. While I extracted and reverse transcribed RNA from the sampled spleen tissue, undergraduate biology student Lucy Addison conducted the template production (for standard curves) and qPCRs assays to quantify the expression of inflammatory cytokines (section 5.3.7). I offer wholehearted thanks to everyone who contributed their time and expertise to this study.

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Keywords

Hippocampal formation, avian brain, adult neurogenesis, chronic stress, keel bone fractures, commercial housing, animal welfare, *Gallus gallus domesticus*

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
AD	Antidepressant
AHN	Adult hippocampal neurogenesis
ANP	Amplifying neural progenitor
ASV	Amplicon sequence variant
BNST	Bed nucleus of the stria terminalis
BrdU	Bromodeoxyuridine
CMS	Chronic mild stress
CORT	Glucocorticoid hormones, including corticosterone and cortisol
CRF	Corticotrophin-releasing factor
DCLK1	Doublecortin-like kinase 1
DCX	Doublecortin
DG	Dentate gyrus
EC	Entorhinal cortex
EE	Environmental enrichment
EPM	Elevated plus maze
FA	Fluctuating asymmetry
FST	Forced swim test
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor
HF	Hippocampal formation
H:L	Heterophil:lymphocyte
HPA	Hypothalamic-pituitary-adrenal
IHC	Immunohistochemistry
IL	Interleukin (1B/6/8/10)
IR	Infrared
KBF	Keel bone fracture
LB	Lohmann Brown (layer hybrid)
LBR	Lamin B receptor
LMM	Linear mixed model
LPS	Lipopolysaccharide
LSL	Lohmann Selected Leghorn (layer hybrid)
MCM2	Minichromosome Maintenance Complex 2

MDD	Major depressive disorder
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NP	“Non-preferred” (housing condition)
NSF	Novelty-suppressed feeding
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
P	“Preferred” (housing condition)
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PVN	Paraventricular nucleus
QNP	Quiescent neural progenitor
qPCR	Quantitative polymerase chain reaction
RFID	Radio frequency identification
SGZ	Subgranular zone
SSRI	Selective serotonin reuptake inhibitor
TGFβ	Tumour growth factor β
TCA	Tricyclic antidepressant
TI	Tonic immobility
TST	Tail suspension test
UCMS	Unpredictable chronic mild stress
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone

Chapter 1. Literature Review

1.1 Introduction

1.1.1 Background & definitions

In recent years, concern for the welfare of farmed animals has become a higher priority among consumers (McKendree *et al.*, 2014), and is of sufficient influence to deter them from purchasing certain products (Alonso *et al.*, 2020). EU citizens have previously identified laying hens as the animals most in need of welfare improvements (European Commission, 2005), and consumers have expressed willingness to pay for legislative improvements in practices surrounding egg production (Bennett, 1996). Historically, proponents of the biological functioning approach have defined welfare in terms of the ability of an animal to "cope" with its environment, and focused upon ensuring that primary biological needs are satisfied (Broom, 1986; Duncan, 2002). Assessment of farm animal welfare has therefore often focused on physical health (e.g. Pritchard *et al.*, 2005) or monitoring resource inputs (Yeates and Main, 2008). In contrast, conceptualisations of welfare from the "feelings school" have emphasised the crucial importance of the affective experience of the animal, rather than the primary needs that these feelings have evolved to protect (Duncan, 2002; Mendl *et al.*, 2010). Generally, the physical and mental state of the animal are accepted to comprise the two key areas covered by welfare considerations (Van Poucke *et al.*, 2007). Recent efforts have highlighted the goal of ensuring that farmed animals lead a life which from their perspective is "worth living" (FAWC, 2009). This notion is compatible with defining good welfare as the absence of negative subjective emotional states, collectively termed "suffering", and the presence of positive subjective emotional states (Duncan, 2002). Operationally, a "life worth living" could be conceived to entail an acceptable overall balance between the positive and negative subjective emotional experiences occurring over an animal's lifespan. Correspondingly, efforts to improve welfare equate to the implementation of husbandry practices to minimise negative experiences and provide opportunities for those which are positive or enriching (Mellor, 2016). A prerequisite for taking such measures is an understanding of which conditions and stimuli are associated with these desirable and undesirable experiences, from the perspective of the animal. Gaining this understanding necessarily depends upon the use of reliable, objective indicators of welfare.

Consciously experienced subjective emotions are presumed to exist for many species of non-verbal animal (Poirier *et al.*, 2019), but cannot be directly assessed (Mendl *et al.*, 2010).

However, indirect measures may provide objective evidence regarding these subjective emotional states (Duncan, 2002). According to certain contemporary frameworks, emotions are categorised according to the fundamental dimensions of arousal and valence (Stanley and Meyer, 2009; Mendl *et al.*, 2010). The dimension of valence indicates whether an experience is negative/punishing or positive/rewarding, while arousal refers to the degree of activation, or intensity, of the associated emotional state (Mendl *et al.*, 2010). A temporal distinction can further be made between short-lived, discrete emotional responses, or transient affective states, and subsequently induced moods. A mood may be defined as a relatively enduring affective state, which is generalised to alter an individual's responses to future events and in different contexts (Nettle and Bateson, 2012). Studies in human subjects suggest that while induced changes in the level of arousal dissipate quickly, induced changes in the valence of affective experiences are maintained for longer durations (Gomez *et al*., 2009), pointing to a closer association with mood. Both enduring moods and recurrent transient affective states have implications for lifetime welfare. To evaluate whether the life of an animal is "worth living", indirect indicators of long-term, or ideally cumulative, experience must be objectively quantified. Cumulative experience can be defined as the net consequence of all events that influence the welfare of an animal over its lifetime, accounting for amelioration or exacerbation of those occurring previously (Poirier *et al.*, 2019).

1.1.2 Current welfare issues for laying hens

Issues considered to be influential for the welfare of commercial laying hens can broadly be categorised as relating either to health, or to behaviour. Skeletal and foot health have been argued to comprise the greatest areas of concern in laying hen welfare, with this being particularly true for ageing birds (Webster, 2004). Associated conditions included keel bone fractures, osteoporosis, bumble foot and toe pad hyperkeratosis (Tauson and Abrahamsson, 1994). Disease and plumage condition also have notable implications for both welfare and production (Hartcher and Jones, 2017). Behaviourally, concerns surround either the restricted expression of natural behaviours under certain housing conditions, or the occurrence of behaviours deleterious for the collective wellbeing of the group or flock. Prevalent behaviours in this latter category include feather-pecking, cannibalism (or feather-pecking leading to mortality), aggressive/agonistic behaviours, piling and smothering (Bright and Johnson, 2011; Hartcher and Jones, 2017).

Housing hens in conventional battery cages was demonstrated to be associated with several negative outcomes for welfare, including restricted movement and behavioural repertoires due

to inadequate space (Widowski *et al.*, 2016). Adverse consequences for musculoskeletal health were also found, as many birds developed disuse osteoporosis and the incidence of fractures at the end of the laying period was very high (Hartcher and Jones, 2017). In the European Union, legislation was passed to phase out the use of barren battery cages (EU Council Directive 1999/74/EC), which have now been prohibited since 2012 (CEC and Communities, 1999). Modern systems are required to provide at least 750 cm^2 space per hen, along with nests, perching space and scratching substrate, to enable pecking and scratching behaviours.

Subsequent to this regulation, commercial laying hens in the EU have been housed either in enriched cages (also referred to as furnished or modified cages), or in aviary or barn systems, some of which provide access to an outdoor range (Lay Jr. *et al.*, 2011). Compared to conventional cages, enriched cages allow increased behavioural expression, but maintain benefits relating to hygiene and efficiency of production (Hartcher and Jones, 2017). Enriched cages are also associated with the lowest incidence of keel bone fractures of any housing alternative (Rodenburg *et al.*, 2008; Hartcher and Jones, 2017). However, enacting a full behavioural repertoire is still not possible (Lay Jr. *et al.*, 2011), as enriched cages can offer only limited opportunities for foraging and ground scratching, behaviour in the vertical plane is restricted, and provision of dustbathing facilities is variable between models (Hartcher and Jones, 2017).

Research has indicated that enriched cages are viewed less favourably by the public than cage-free systems (Norwood and Lusk, 2011), while many retailers have recently pledged to sell only cage-free eggs (Lusk, 2018). While cage-free systems do potentially allow for a full behavioural repertoire (subject to management practices and the furniture provided), different welfare issues associated with this alternative mean that welfare in cage-free systems is currently highly variable (Hartcher and Jones, 2017). Feather pecking may be more severe and harder to control in cage-free systems, due to larger flock sizes and spread of the behaviour by social learning (Cloutier *et al.*, 2002; Lay Jr. *et al.*, 2011). This issue is often combatted by trimming the beaks of day-old pullets, but this procedure is known to be painful for the birds (Gentle *et al.*, 1990; Van Liere, 1995). Levels of cannibalism are subsequently higher in non-cage systems than in enriched cages, as are rates of mortality through infectious diseases (Fossum *et al.*, 2009; Weeks *et al.*, 2012). The incidence of keel fractures during the laying period is also greater in non-cage systems (Rodenburg *et al.*, 2008; Lay Jr. *et al.*, 2011).

Free-range systems are generally perceived to be associated with the highest welfare, and the public value produce from free-range laying hens most highly (Norwood and Lusk, 2011).

However, there is a large degree of individual variation in the extent to which hens make use of the external areas provided, and some birds never opt to venture outside (e.g. Richards *et al.*, 2011; Gebhardt-Henrich *et al.*, 2014; Campbell *et al.*, 2019). Lack of cover (Lubac and Mirabito, 2001; Dawkins *et al.*, 2003; Zeltner and Hirt, 2008) and weather conditions which are cold, wet or windy (Stadig *et al.*, 2017) may present barriers to outdoor ranging.

Generally, the relative influence of different issues detrimental to laying hen welfare varies between production systems and with management practices, and there is a need for objective methods of comparing the cumulative affective experience for hens that is associated with each alternative. While each system has its own drawbacks (Lay Jr. *et al.*, 2011), it is often unclear which of the potentially stressful experiences and conditions are most salient or negative from the perspective of the birds.

1.1.3 Existing methods of assessing poultry welfare

In commercial settings, welfare is often assessed at the level of environmental-based parameters, which involves monitoring and managing those environmental conditions (e.g. temperature, humidity, ammonia, light levels) and resource inputs (e.g. feed, water, substrates) that are prerequisites for acceptable welfare (Mollenhorst *et al.*, 2005). Other assessments are based upon direct measures of animal-based parameters. In this context, animal welfare is usually evaluated by measuring the animal's responses to different environments or procedures. As animal welfare is generally accepted to cover both the physical and emotional state of an animal (Duncan and Dawkins, 1983; Fraser, 1995; Van Poucke *et al.*, 2007), a range of physiological, behavioural and clinical indicators are typically compared between groups that have been housed, or otherwise treated, differently (Nicol *et al.*, 2009). Estimates of production, health/pathology, behaviour, physiology and immunology therefore contribute to welfare assessment (Van Poucke *et al.*, 2007).

1.1.3.1 Production measures

Because poor welfare often reduces productivity (Blokhuis *et al.*, 2013) and chronic stress may lead to delayed or reduced laying in commercial hens (Shini *et al.*, 2009), indices of production are often cited during the assessment of welfare. Such parameters include hen-day egg production and egg weight (e.g. Hemsworth and Barnett, 1989). Measurements of egg quality may also be taken, as irregularities or defects in the shell can result from delays in oviposition due to stress (Hughes *et al.*, 1986; Mazzuco and Bertechini, 2014). Other factors with direct implications for commercial profitability, such as cumulative mortality, are also frequently

recorded. Feed consumption may also reflect stress, in terms of the metabolic demands of restoring or maintaining homeostasis. For example, hens with poor feather cover have impaired thermoregulatory capacities, and exhibit increased feed intake to compensate for this inadequate insulation (Tauson and Svensson, 1990).

1.1.3.2 Physiological measures

Physiological evaluations of animal-based welfare often comprise direct assessments of the stress response. Indeed, definitions of animal welfare relating to biological functioning, which focus upon the attempt of an individual to cope with its environment, are strongly related to the state of stress (Duncan, 2002). Stress can be defined as an integrated response elicited by real or perceived threats to homeostasis or wellbeing, which comprises a collection of physiological, behavioural and emotional processes acting to restore equilibrium (Herman *et al.*, 2016). The environmental variables causing these disruptions to the homeostatic balance, and necessitating coping, are termed stressors (Broom, 2001). The stress response is activated by multiple modalities of stressor, including homeostatic or systemic stressors, such as deviations in levels of blood glucose or body temperature, physical insults or injuries, and psychogenic or anticipatory stressors, such as social challenge or the perception of threat (Anisman *et al.*, 2001). Internal responses are primarily coordinated through activation of the sympathetic division of the autonomic nervous system and of the hypothalamic-pituitary-adrenal (HPA) neuroendocrine axis (Ziegler, 2004), with myriad downstream effects. In birds, corticosterone is the principal glucocorticoid hormone released by the adrenal gland as the end-product of HPA-axis activation (Shini *et al*., 2008). The primary adrenal glucocorticoid differs between species (e.g. corticosterone in birds, rats, mice and amphibians; cortisol in humans, many other mammals, and fish (Senft *et al.*, 2016)), but these compounds mediate comparable functions and are hereon collectively termed CORT.

While the acute experience of stress is accompanied by a transient affective state of high arousal and negative valence (Feldman *et al.*, 1999), the response facilitates coping and elicits behaviours critical for survival (Mellor, 2016). Completely eliminating this experience from the lives of farmed animals is therefore probably not an attainable or desirable goal (Mellor, 2016). Measures of the acute stress response applied to poultry, such as heart rate and the quantification of surface temperature changes via infrared thermography (Weimer *et al.*, 2020), will therefore not be discussed here. However, while short-term or acute stress has a duration of minutes to hours, prolonged or recurrent sources of stress may lead to chronic activation of the stress response, persisting for several hours a day over a period of weeks (Dhabhar and

McEwen, 1997). When maintained on a long-term basis, the redistribution of resources mediated by the stress response leads to an impairment of biological functions relating to immunity, reproduction and growth (Rich and Romero, 2005; Alm, 2015). In circumstances of repeated stress or dysregulation of the HPA-axis, CORT generally promotes pathophysiology (McEwen, 2006), and is associated with psychological disorders of anxiety, depression and panic in human subjects (Broom, 2001). The damaging actions of CORT under conditions of chronic stress have been termed "allostatic load", which refers to the cost of adaptation to consistently adverse conditions incurred by the body (McEwen, 2006). In the context of animal welfare, chronic stress may also be conceptualised as a "failure to cope" with these unfavourable conditions (Broom, 2001).

Responses of the HPA-axis and sympathetic nervous system to stress, and the associated immunological changes (Ziegler, 2004; Alm, 2015) have quantifiable proxies. Despite sustained concerns regarding its validity, CORT concentration in the blood remains the most widely employed physiological measure of laying hen welfare (Nicol *et al.*, 2009), while CORT levels can also be measured in faecal metabolites, and in the yolk and albumen of eggs (Alm, 2015). CORT concentrations in plasma begin to rise minutes after exposure to an acute stressor (Chloupek *et al.*, 2011), and peak after around 30-50 minutes after commencement of stress (Droste *et al.*, 2008). Chronic stress usually leads to chronically elevated concentrations of CORT at baseline, while facilitation of the HPA-axis occurs to compensate for negative feedback mechanisms and maintain responsiveness to acute stressors, often leading to stress-induced CORT secretion that is also greater in magnitude (Sakellaris and Verkinos-Danellis, 1975; Marti *et al.*, 1994; Rich and Romero, 2005). These indices of HPA-activity are thus often used as indicators of longer-term stressful experience.

However, findings relating various sources of stress to plasma CORT levels in laying hens are highly inconsistent (Thaxton, 2004; Shields and Duncan, 2009). Notably, CORT levels do not reflect conditions leading to increased mortality, such as decreasing space allowance in conventional cages (Rushen, 1991; Shields and Duncan, 2009). CORT secretion is episodic in nature, exhibiting circadian and ultradian rhythms (Droste *et al.*, 2008), and its role in metabolism means that basal levels are influenced by extraneous factors such as diet (Pal *et al.*, 2015). Effects of chronic stress on activity of the HPA-axis are complex, and it is not clear whether changes in basal CORT or in the nature of secretory episodes are more important or informative (Rushen, 1991). Moreover, the acute nature of the latter response means measurements are susceptible to the influence of handling stress during sample collection (Mench, 1986). Furthermore, while CORT secretion reflects arousal, the response is not

valence-specific. As such, plasma CORT is also elevated by positive experiences, such as sexual activity (Leuner *et al.*, 2010), exercise (Stranahan *et al.*, 2006) and social housing (Martin and Brown, 2010) in mice. Concurrent measurement of a valence-specific marker may therefore be necessary for valid interpretation of findings.

As well as being recruited by infection, leucocyte white blood cells are responsive to non-infectious stress (Maxwell, 1993). Two major subgroups of leucocytes in birds are heterophils and lymphocytes. While heterophils fulfil a phagocytic role in the acute inflammatory response, lymphocytes are involved in adaptive immunity (Alm, 2015). Initially, adding CORT to the feed of domestic chickens was found to decrease the number of lymphocytes and increase the number of heterophils in blood samples (Gross *et al.*, 1980). In addition to being less variable than individual (heterophil or lymphocyte) cell numbers alone, this heterophil:lymphocyte (H:L) ratio proved to be a more reliable indicator of levels of CORT in the feed than quantified levels of CORT in plasma (Gross and Siegel, 1983). The H:L response was subsequently shown to be initiated by a range of different stressors, including environmental, psychological and nutritional stress (Maxwell, 1993; Davis *et al.*, 2008). For example, H:L ratios were elevated by exposure to heat or cold, handling or induction into tonic immobility, and poor litter conditions (reviewed in Thaxton, 2004). Generally, H:L has been accepted as a good measure of the chicken's perception of stress in its environment (Gross and Siegel, 1983), which is a more reliable indicator of the experience of mild to moderate stress than plasma CORT (Maxwell, 1993; Thaxton, 2004). It also appears sensitive to more subtle stressors (Müller *et al.*, 2010).

Generally, the H:L ratio is utilized as a measure of relatively long-term stressful experience. However, studies have also reported fairly rapid increases in H:L ratio following short-term stressors or CORT treatment. Firstly, chickens given dietary CORT exhibited a linear increase in H:L ratios 24-hours after this treatment began (Shini *et al.*, 2008). Secondly, subjecting chickens to a loud sound for just 30 seconds produced a rise in H:L ratios 18 hours later, which peaked after 20 hours and returned to pre-stress values after 30 hours (Gross, 1990). Thirdly, three to four hours of road transport significantly increased H:L ratios in broiler chickens (Mitchell *et al.*, 1992). Finally, broiler H:L ratios were elevated by 12 hours of heat stress (Mashaly *et al.*, 2004). When used to assess longer-term, cumulative experience, quantified H:L levels may therefore disproportionately reflect recent, acute stress. It has also been suggested that the proxy is too simplistic, as stress may produce atypical forms of leucocyte while the H:L ratio remains low (Cotter, 2015). This observation may relate to differential white

blood cell responses to severe stress, for which H:L ratios are not considered to be a reliable indicator (Maxwell, 1993).

1.1.3.3 Phenotypic measures

Phenotypic qualities are also often assessed as indicators of long-term hen welfare. Feather cover is widely scored (Freire and Cowling, 2013; Alm, 2015) and was ranked as the most important single indicator of hen welfare in a survey of experts (Rodenburg *et al.*, 2008). Factors known to affect feather cover include stocking density, cage material, feather pecking, hen age, feed-protein ratio, temperature and light levels (reviewed in Yamak and Sarica, 2012). Certain behavioural evidence suggests that poorly feathered hens are more fearful then their well-feathered conspecifics (Ouart and Adams, 1982; Na-Lampang and Craig, 1990). Similarly, indices of bone health (e.g. fracture status/severity, strength, mineralisation) (Rufener *et al.*, 2018) and damage to the feet (lesions, necrosis) (Tauson *et al.*, 2005) are often commonly scored, along with parasite burdens (e.g. through faecal egg counts or post-mortem examination of the gastrointestinal tract) (Bennett *et al.*, 2011). As these measures reflect discrete aspects of a hen's health, it may be necessary to take several in order to produce a complete picture of physical welfare.

Fluctuating asymmetry (FA) refers to small, randomly-directed deviations of symmetry in bilateral traits, and is the most commonly used index of developmental instability (Møller and Manning, 2003; Van Poucke *et al.*, 2007). Constraints on energy allocation mean that it is more difficult to maintain levels of developmental stability under conditions of high environmental stress, and FA is the manifestation of deteriorated developmental homeostasis in adult morphology (Parsons, 1990). FA has been proposed as an integrated measure of animal welfare, because it constitutes an objective reflection of an individual's ability to cope with the sum of challenges faced during its development (Møller and Manning, 2003). However, results of studies linking FA with farm animal welfare are mixed, while stressors impacting the physical state of the animal during rearing were more likely than emotional stressors to induce an effect on FA (reviewed in Tuyttens, 2003). A greater impact of unpredictable feed restriction compared to predictable feed restriction on FA in juvenile starlings (Swaddle and Witter, 1994) suggested that subjective experiences may also influence this outcome measure, but there is little other empirical evidence (Tuyttens, 2003). Moreover, FA did not differ from control birds for *ad libitum* fed broiler chickens exposed experimentally to several forms of stress (pain and frustration, or wet litter, high temperature and stocking density) (Van Poucke *et al.*, 2007). It was concluded either that FA is not a suitable measure for fast-growing broiler chickens, or that

it is not a sensitive indicator of welfare for any *ad libitum*-fed farmed animals, due to an absence of constraints on energy allocation. While developmental instability reflects the ability of growing animals to cope with their environment, the measure has little promise as an indicator of welfare after growth processes are completed (Tuyttens, 2003), and is therefore unlikely to reflect stress experienced by adult laying hens during the laying period. FA levels have also been noted to not demonstrate consistent associations with tonic immobility durations and H:L leukocyte ratios, across breeds of chicken or when different bilateral traits are measured (Campo *et al.*, 2000; Campo *et al.*, 2002), suggesting they may not constitute a general tool to address fear and stress susceptibility in birds that have not been experimentally stressed.

1.1.3.4 Behavioural measures

Behavioural assessments in poultry may involve observing the natural (i.e. non-manipulated) behaviours of birds in their home environment and recording their frequencies with respect to an ethogram (Alm, 2015), for example for comparison between housing systems. A common behavioural assessment approach is to compare behaviour in commercial or laboratory settings to that in a natural or ideal environment. However, in the case of domestic chickens, this method is complicated by genetic selection that has altered the behavioural characteristics of modern strains from those of wild or feral hens, while not all "natural" behaviours need to be performed for good welfare (Dawkins, 2003; Weeks and Nicol, 2006). Another approach focuses upon detecting signs of poor coping, identified under experimental situations, in environments of interest. "Abnormal" behaviours with no apparent purpose or function have been identified as indicators of negative states in laying hens, relating to fearfulness, aggression, frustration and deprivation (Weeks and Nicol, 2006). These behaviours are variously thought to constitute time fillers, displacement activities and approximations or re-directions of natural behaviours that are impossible to perform in a particular setting (Cooper and Albentosa, 2003; Weeks and Nicol, 2006). Examples include stereotypic pacing, spot-pecking, yawning, headshaking, feather-pecking and sham-dustbathing (Blokhuis *et al.*, 1993; Weeks and Nicol, 2006). Measuring the frequency of such behaviours may be informative as to the relative welfare of groups of hens. For example, feather-pecking is generally believed to be re-directed foraging behaviour (Nicol *et al.*, 2001), and was reduced with the provision of foraging material, in accordance with other indicators of improved welfare (El-Lethey *et al.*, 2000).

Behaviour during particular test scenarios is also commonly measured, with the majority of these tests designed to estimate the fearfulness of the birds (Alm, 2015). While fear can be defined as an adaptive psychophysiological response to perceived danger (Jones, 1986),

stressful experiences are generally thought to increase the frequency, magnitude or duration of fear behaviours. Fearfulness is commonly defined as a response to a known threat, whereas anxiety constitutes a response to an unknown threat, but both states may have similar negative associations for welfare (Campbell *et al.*, 2019).

In many species of animal, an unlearned tonic immobility (TI) response is elicited by a brief period of physical restraint, and comprises a catatonic-like state of immobilisation and reduced responsiveness to external stimulation (Jones, 1986). This reaction is particularly robust and easy to discern in domestic fowl, and may persist for anywhere between a few seconds and several hours after restraint is terminated (Jones, 1986). TI is widely used as a method of estimating fearfulness, and there is substantial evidence for the positive relationship between the antecedent fear state and the duration of TI. Multiple procedures intended to increase fear, such as electric shocks, suspension over a visual cliff, simulated predatory encounters and loud noises, prolong the TI reaction, whereas duration of the response is attenuated by factors that reduce fear, including taming, habituation, administration of tranquillisers, the presence of social companions and conditioned safety signals (Gallup, 1974a; Jones, 1986). Individual chicks that exhibited long durations of TI were also likely to demonstrate high levels of fear in other contexts, such as exposure to a novel environment or a loud noise (Jones, 1986; Jones and Mills, 2003). Increases in arousal unrelated to fear, such as those produced by food deprivation or the administration of amphetamine, either failed to affect TI or diminished the reaction (Gallup, 1974a), indicating that the response is perpetuated in accordance with negatively valenced affect. There is thus plentiful evidence that durations of TI in domestic chickens are responsive to acute stress. It was also found that chronic infusion of physiological levels of CORT prolonged TI reactions (and increased H:L ratios) in adult White Leghorn hens, suggesting that chronic stress might also predispose chickens to greater behavioural fear responses (Jones *et al.*, 1988). Indeed, housing under conditions of continuous light for 16 weeks prolonged TI durations across 11 breeds of chicken (Campo *et al.*, 2007). Six weeks of environmental enrichment also decreased TI durations in broiler chicks relative to control birds (Yildirim and Taskin, 2017), suggesting that the behaviour also reflects experiences with a positive valence.

Durations of TI have been noted to differ between hens from different commercial housing systems (Jones and Faure, 1981; Hansen *et al.*, 1993; Scott *et al.*, 1998), with cage tier in intensive systems (Jones, 1985), and with varying use of outdoor areas in free-range systems (Hartcher *et al.*, 2016). However, other evidence relating TI to long-term welfare is more mixed. For example, whereas H:L ratios were elevated after 72 hours of fasting or frustration of

feeding, TI responses were not affected by these stressors (Jones, 1989). TI responses also do not always vary in a direction consistent with other measures of stress. For example, a (brown) strain of chicken with high H:L ratios (indicative of stress) exhibited shorter durations of TI than a (white) strain with low H:L ratios (Mahboub *et al.*, 2004). Broiler chickens housed with access to an outdoor run were more active, enacted more natural and comfort behaviours, and had lower FA than indoor housed birds, but also exhibited longer durations of TI than indoor-only birds (Zhao *et al.*, 2014). TI has also been determined to have a large heritability component in chickens, with genetic strains of birds differing in their responses (Gallup, 1974b). Individual differences in TI durations within a strain are also notable (Jones, 1989; Jones and Mills, 2003) and heritable (Gallup *et al.*, 1976; Nakayama *et al.*, 2010). Circumstances relating to testing may additionally influence TI durations, as latencies were prolonged by the novelty of the experimenter wearing an unfamiliar coat, even when birds were tested in their familiar home environment (Jones, 1984). Generally, it may be difficult to interpret the extent to which TI reflects long-term experience versus acute situational fearfulness surrounding testing.

Other common behavioural tests of fearfulness in poultry include activity in an open field and avoidance of a novel object, or of humans (Hughes and Black, 1974; Hemsworth and Barnett, 1989; Graml *et al.*, 2008; Campbell *et al.*, 2019). In each case, a greater duration of freezing and avoidance is indicative of a higher level of fear. Individual differences between hens also exist in movement in an open-field and latency to feed in a novel testing environment (Campbell *et al.*, 2019). Some behavioural tests (e.g. attention bias to an alarm call (Campbell *et al.*, 2019) and avoidance of humans (Graml *et al.*, 2008)) may not be suitable for the most fearful birds, as these individuals often remain frozen and fail to perform the measured behaviour in the allocated time, leading to their subsequent exclusion and the omission of data (Campbell *et al.*, 2019).

Another behavioural approach is to measure animal decision making, using tests of relative preference for discrete options, or consumer demand methods that measure how hard an animal is willing to work to access a particular resource. As an assessment of motivation, the latter method typically measures whether the use of the resource is defended when access is made more difficult or demands more energy (i.e. a cost is imposed) (Weeks and Nicol, 2006). Under the assumption that animals can weigh the relevant options and select in their own best interests, choice may be considered a particularly meaningful measure of welfare (Nicol *et al.*, 2009), with implications for optimising commercial conditions. Multiple studies have made use of simple choice tests for determining preferences for types of foraging materials, lighting, food,

and social companions, etc. (Dawkins, 1982; Nicol, 2011). Tests of behavioural priority have pointed to key factors for inclusion in enriched commercial housing systems, such as perches, enclosed nest boxes and foraging materials (Bubier, 1996; Olsson and Keeling, 2002; Cooper and Appleby, 2003). However, there are certain interpretational issues. In the case of preference tests, meaningful results depend upon the experimenter presenting valid and comparable alternatives, while it is not clear if the animal is choosing between groups of high value or low value options, meaning the preference revealed is always relative (Duncan, 1978). It is generally difficult to demonstrate unequivocally that animals have a specific representation of a resource goal, while the extent to which they consider the long-term consequences of their decisions is unclear (Weeks and Nicol, 2006). It is also possible for information gathering, monitoring and patrolling activities to be misconstrued as active choice (Nicol *et al.*, 2009). Small procedural changes have been noted to sometimes have dramatic effects on the outcomes of choice or demand tests (Weeks and Nicol, 2006). For example, preferences and behavioural priorities may differ depending on whether the animal can see the resource it is working to obtain (Warbuton and Mason, 2003). Careful consideration of experimental design may be crucial in such explorations.

Studies have also identified vocalisations made by chickens during experimental situations designed to provoke negative affect. For example, a call associated with frustration is made after thwarting of feeding, dust-bathing or normal pre-laying behaviour, and when a behaviour ceased to be rewarded in an operant conditioning paradigm (reviewed in Manteuffel *et al.*, 2004). Distress calls are elicited by isolation in chicks (Lehr, 1989), and also by the administration of peptides involved in initiation of the stress response (Panksepp and Normansell, 1990). The rate of distress vocalisations is reduced with prolonged isolation, but maintained in chicks treated with antidepressant drugs (Lehr, 1989), suggesting that the former decline reflects a depressive-like helplessness response, rather than positive habituation or coping (Manteuffel *et al.*, 2004). Measuring vocalisations in commercial settings may therefore provide a proxy for negative states of fear and frustration in poultry. For example, acoustic properties of distress calls in flocks of broiler chicks were found to predict production- and welfare-relevant outcome measures later in life (Herborn *et al.*, 2020). However, vocal responses of adult birds appear to differ markedly at the individual (Richard-Yris *et al.*, 1998) and strain (Zimmerman and Koene, 1998) levels (Manteuffel *et al.*, 2004). Whereas Brown Warren strain hens exhibited a vocal reaction to the frustration of non-reward, White Leghorn hens made more distress calls throughout the study, and did not react differently in the rewarded

and non-rewarded conditions (Zimmerman and Koene, 1998). Such variation may complicate the interpretation of acoustic assessments.

1.1.4 Need for novel measures of long-term experience

As discussed, many of the existing measures employed in assessments of laying hen welfare are not free from methodological limitations or interpretational issues. This has led to the conclusion that valid, sensitive and reliable animal-based indicators, which can feasibly be measured or scored on-farm or at slaughter, are lacking (Van Poucke *et al.*, 2007). Given that there is no single marker of absolute good or poor welfare, it is increasingly argued that measurement of a broad range of the available indicators is required (Mason and Mendl, 1993; Fraser *et al.*, 1997; Spoolder *et al.*, 2003; Nicol *et al.*, 2009). However, there is no consensus about exactly which, or how many, parameters should be measured, while quantification or scoring according to different scales makes integration difficult (Tuyttens, 2003). Moreover, when multiple measures are taken, these indicators do not always co-vary in consistent ways (Nicol *et al.*, 2009). Attempts to deal with this issue have mainly devised schemes for assigning differing relative weightings to various indicators, and drawn conclusions based on integration of the weighted parameters (Spoolder *et al.*, 2003; Nicol *et al.*, 2009). These weightings are usually determined by surveying expert opinion (e.g. Bracke *et al.*, 2002), though the aforementioned problems mean that these experts may have little objective evidence to inform their opinions (Nicol *et al.*, 2009).

Many of these problems would be ameliorated by the identification of sensitive, integrated measures of long-term welfare. An integrated measure can be defined as a single indicator in which the retrospective contributions of different stressors, as perceived by the individual, have already been collated (Tuyttens, 2003). While FA might potentially comprise an integrated measure of the total sum of stress experienced by an organism during development, it is not applicable to fully grown animals, such as adult laying hens (Tuyttens, 2003). Moreover, an ideal proxy measure for retrospective assessment of the cumulative experience of farmed animals would also integrate positively-valenced subjective experiences, along with negative stress. While the objectivity of FA as a measure is supported by the existence of an optimum value (i.e. 1, reflecting perfect symmetry), this property also imposes a ceiling effect, whereby it is not possible for positive experiences indicative of good welfare to lead to additional improvements in the measure. As such, a biomarker capable of integrating an animal's relative experience of chronic stress versus positive moods on a long-term basis would facilitate the assessment of quality of life. Were this measure also quantitatively sensitive to the duration or

magnitude of those experiences, its application would contribute much to the understanding and improvement of laying hen welfare. Assessment of the long-term efficacy of interventions designed to improve welfare, such as provision of access to an outdoor range, would also be aided by such a biomarker. This research project will explore the use of a putative novel welfare indicator with these properties, as applied to commercially relevant conditions for laying hens.

1.2 Physiological Correlates of Experience

1.2.1 The hypothalamic-pituitary-adrenal axis

As briefly discussed, the hypothalamic-pituitary-adrenal (HPA) axis is one of the primary effector systems of the physiological stress response (Tsigos and Chrousos, 2002). Upon perception of a stressor, neurons of the paraventricular nucleus (PVN) of the hypothalamus secrete stimulus-specific combinations of releasing factors into the hypophyseal portal system (Jacobson and Sapolsky, 1991). The main secretagogues involved have been identified as corticotrophin releasing factor (CRF), arginine vasopressin (or congener arginine vasotocin in birds (Rich and Romero, 2005)), oxytocin and epinephrine (Jacobson and Sapolsky, 1991). Under non-stressful conditions, CRF and vasopressin are both released according to circadian and ultradian rhythms, with an average of two to three pulsatile secretory episodes occurring each hour (Engler *et al.*, 1989), and increasing in amplitude in the early morning (Tsigos and Chrousos, 2002). Following transportation through the hypophyseal portal system to the anterior pituitary gland, these peptide factors synergistically stimulate the release of adrenocorticotropin hormone (ACTH) into the bloodstream (Jacobson and Sapolsky, 1991). In turn, circulating ACTH stimulates CORT release from the adrenal cortex. Under resting conditions, levels of ACTH and CORT in the general circulation exhibit diurnal and pulsatile rhythms corresponding to their upstream mediators (Tsigos and Chrousos, 2002).

Glucocorticoid hormones are the final effectors of the HPA-axis, and coordinate whole body homeostasis and multi-organ responses to stress (Tsigos and Chrousos, 2002). In particular, CORT brings about metabolic changes, including mobilization of glucose to the muscles, concurrent with the suppression of long-term processes such as reproduction and growth (Sapolsky *et al.*, 2000). These changes are accomplished through the activation of intracellular (cytoplasmic) or membrane-bound glucocorticoid receptors. Target cells express intracellular mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), to which CORT binds to exert transcriptional effects that facilitate coping with stressors (Senft *et al.*, 2016). Mechanistically, the CORT-bound receptor-ligand complex enters the nucleus, where it forms a homodimer and interacts with specific hormone response elements to initiate the transcription

of target genes (Senft *et al.*, 2016). The complete network of genes with MR- or GR-dependent transcription has not been fully characterised, but over 300 target genes with GR-bound promoters were identified in the mouse liver, including genes for other transcriptional regulators (Phuc Le *et al.*, 2005). The regulatory networks involved thus appear to be pervasive.

In order to limit the duration of tissue exposure to CORT and minimise its catabolic, anti-reproductive and immunosuppressive effects, acute stress-induced CORT release is inhibited through a collection of negative feedback mechanisms that terminate the ACTH secretory response (Jacobson and Sapolsky, 1991; Tsigos and Chrousos, 2002). CORT plays a key role in these regulatory processes, principally through binding to GR in the hypothalamus and pituitary gland, with fast-feedback initiated at receptors on the CRF neurons of the PVN (Herman *et al.*, 2012), but also through actions at extra-hypothalamic centres (primarily within the limbic system) (Tsigos and Chrousos, 2002; Senft *et al.*, 2016). CORT binding to MR and GR additionally mediates the basal circadian activity of the HPA-axis under non-stress conditions (Jacobson and Sapolsky, 1991; Tsigos and Chrousos, 2002). Throughout the diurnal cycle, circulating concentrations of CORT range from 0.5 to 50 nmol/l, but these may exceed 100 nmol/l in response to stress (De Kloet, 1991). Compared to GR, MR (also known as type II receptors) possess a higher affinity for CORT (five to ten-fold greater (Herman *et al.*, 2005)) and are saturated at low concentrations. As such, MR primarily mediate basal levels of circulating CORT throughout the circadian cycle, whereas lower-affinity GR (also known as type I receptors) are bound only during periods of high (i.e. stress-induced) CORT concentrations, and modulate the majority of negative feedback mechanisms to terminate the stress response (Barden, 2004; Herman *et al.*, 2012). Whereas GR are widely distributed throughout the brain, MR are expressed in relatively few brain areas of the mammalian brain, and are absent from the PVN. Indeed, while MR are possibly present in certain brainstem nuclei, these receptors are largely confined to the limbic system (Jacobson and Sapolsky, 1991).

1.2.2 Regulation of the HPA-axis by the hippocampus

Both MR and GR are expressed within the hippocampus, a limbic structure of the mammalian medial temporal lobe. which acts as a key regulatory of the HPA stress response (Jacobson and Sapolsky, 1991; Senft *et al.*, 2016). Indeed, the hippocampus has been demonstrated to influence the circadian CORT rhythm, the HPA response to stress, and the negative feedback inhibition to terminate this response (Jacobson and Sapolsky, 1991). Firstly, hippocampal lesions in both mammals and birds reduce diurnal variations in plasma CORT, generally by raising nadir concentrations towards those of the peak (Bouillé and Baylé, 1973; Jacobson and

Sapolsky, 1991). In contrast, electrical stimulation of the hippocampus under basal conditions is sufficient to reduce circulating CORT levels (Herman *et al.*, 2012; Bouillé and Baylé, 1973). Secondly, hippocampal stimulation inhibits CORT secretion induced by several stressors (Kawakami *et al.*, 1968; Dupont *et al.*, 1972). Hippocampal ablation also reverses the attenuation of CORT responses to ether stress produced by prior lesions to remove inputs from regions that stimulate the HPA-axis (the amygdala or reticular formation lesion), pointing to the strength of hippocampal inhibition in the intact brain (Knigge and Hays, 1963). Thirdly, the hippocampus is involved in recovery from stress-induced activity of the HPA-axis, and the effects of lesions are most pronounced during this phase (Herman *et al.*, 2005). Indeed, hippocampal lesions in rats induce CORT hypersecretion following the acute psychogenic stress of restraint or exposure to an open field (Herman *et al.*, 1998), and levels remain elevated after delays wherein CORT has returned to baseline in control animals (Sapolsky *et al.*, 1984; Jacobson and Sapolsky, 1991). The contribution of CORT binding to hippocampal GRs is supported by the finding that a transgenic mouse with GR deleted specifically in the hippocampus, cerebral cortex and basolateral amygdala (but not in the central or medial amygdala or PVN) exhibit delayed termination of HPA-axis responses to psychogenic, but not systemic, stressors (Boyle *et al.*, 2005). Generally, the hippocampus appears to be more involved in inhibiting responses activated by stressors associated with the perception of potential threat (e.g. restraint or exposure to open spaces), as compared to immediate threats to homeostasis and survival (e.g. hypoxia) (Herman *et al.*, 2005).

Rather than projecting directly to the HPA effector neurons of the PVN, projection pathways from the hippocampus, cortex and amygdala overlap in various subcortical regions, which appear to constitute relay sites for the integration of limbic information (Herman *et al.*, 2005). As such, the influence of the limbic system on the HPA-axis results from the patterning of responses to the given stimuli in each region, and the relative contributions of the various (excitatory and inhibitory) structures determines the magnitude of the secretory response (Herman *et al.*, 2005). Dysfunction of the hippocampus contributes to an impaired HPA response in aged rats (Sapolsky *et al.*, 1986), and is implicated in the pathophysiology of affective disorders (Campbell and MacQueen, 2004).

1.2.3 Neural plasticity

As discussed, high animal welfare can be defined as the absence of negative affective states (or suffering), and the presence of positive affective states (Duncan, 2002). As these emotions are generated within the brain (Damasio *et al.*, 2000), this tissue may comprise a likely region in

which to find candidate biomarkers of subjective experience. Indeed, experiences are known to induce measurable structural changes in areas of the brain involved in the production and regulation of affect (e.g. as reviewed in McEwen, 2005). Some of these are examples of neural plasticity, or the reciprocal interaction between the structure and function of the central nervous system (Freund *et al.*, 2013). Broom (2001) noted that while changes within the brain regulate bodily coping responses (to stress), adrenal changes also have several consequences for brain function. As exposure to chronic stress can lead to the structural and functional remodelling of neuronal networks (Lupien *et al.*, 2009; Vialou *et al.*, 2013), it is hypothesised that welfare is reflected by plasticity in regions of the brain that are sensitive to experience.

Interestingly, the hippocampus is a site of notable post-developmental plasticity in the mammalian brain (e.g. Altman (1963)). As a key regulator of the HPA stress response that strongly expresses MR and GR receptors, the hippocampus also exhibits a marked reciprocal sensitivity to stress (Joo Kim *et al.*, 2015). Prolonged stress influences multiple indices of hippocampal function, including hippocampal-dependent behaviours and cognition, synaptic plasticity, neuronal firing properties and morphologies, hippocampal volume, and the generation and survival of new hippocampal neurons during adulthood (reviewed in Joo Kim *et al.* (2015). Crucially, aspects of hippocampal plasticity are correlated with both self-reported mood in humans (McEwen, 2005; Miller and Hen, 2015) and behavioural indicators of mood in other species (Jayatissa *et al.*, 2006; Schloesser *et al.*, 2010), according to the valence of the emotion. As measures of cumulative affective experience in mammalian species, indices of hippocampal plasticity have been attributed construct, content and criterion validity (Poirier *et al.*, 2019). In particular, measures of the genesis of new hippocampal neurons in adult mammals and birds exhibit relationships with long-term experience that point to their potential utility as novel proxy indicators of subjective welfare. The following sections will therefore present a detailed examination of this association, with the view of utilising adult hippocampal neurogenesis as an integrated neural biomarker of experience in laying hens.

1.3 The Mammalian Hippocampus

1.3.1 Location & structure

In the mammalian brain, the hippocampus is a collection of densely packed neurons located in the caudo-medial region of the dorsal pallium, below the lateral ventricle (Anand and Dhikav, 2012; Gupta *et al.*, 2012). The primary structure consists of the *Cornu ammonis* (CA, or Ammon's horn) region and the dentate gyrus (DG) (Gupta *et al.*, 2012). The term hippocampal formation (HF) is often used to connote the wider functional system that also includes the entorhinal cortex (EC) and subiculum (Andersen, 2007). Viewed in a transverse section, the HF

resembles two interlocking, mirrored "C" shapes, the superior of which begins adjacent to the EC and subiculum and comprises the pyramidal cell fields of the CA, designated sequentially as CA1 to CA4 (Jacobson and Sapolsky, 1991). The second C-shaped structure is the DG, which is partially surrounded by, and inverted relative to, Ammon's horn. The DG consists of three layers. The outer molecular layer is relatively free of cells, but contains both the dendrites of granule cells and fibres of the perforant pathway (further detailed below) (Amaral *et al.*, 2007). Adjacent is the middle, principle cell layer, known as the granule cell layer (GCL), which consists primarily of densely packed granule cells and ranges from four to eight neurons in thickness in the adult rat brain (Amaral *et al.*, 2007). The GCL encloses the inner polymorphic cell layer, which is alternatively known as the hilus, or hilar region (e.g. Mandyam, 2013). The length of the border between the GCL and polymorphic layer is separated by an area known as the subgranular zone (Mandyam, 2013), sometimes also characterized as the inner third of the GCL (Wu *et al.*, 2014). While the polymorphic layer contains a number of cell types, the most notable are the mossy cells (Amaral *et al.*, 2007).

The flow of information through intrinsic hippocampal circuits is largely unidirectional, and forms an excitatory transverse loop through the subfields (Moser and Moser, 1998; Andersen, 2007). The EC is both the major input and output structure of the HF (Witter *et al.*, 2017). Neurons in EC layer II first project to the molecular layer of DG and to CA3 via the perforant pathway, while neurons in layer III project to CA1 and the subiculum via the perforant and alvear pathways (Andersen, 2007). Mossy fibres originate from the granule cells of the DG and terminate both within the adjacent polymorphic layer and in CA3 (Amaral *et al.*, 2007). Pyramidal neurons in CA3 project to CA1 via Schaffer collaterals, while the pyramidal cells in CA1 project to the subiculum. Finally, both CA1 and the subiculum project back to the deep layers of the EC (Andersen, 2007).

1.3.2 Functions & their segregation across the septo-temporal axis

The hippocampus serves a diverse range of functions, with various roles in spatial cognition, episodic memory (Treves *et al.*, 2008), and affective regulation (Fanselow and Dong, 2010), including the mediation of the HPA stress response detailed in section 1.2.2. In the mammalian brain, these different hippocampal functions are to an extent topographically segregated (Moser and Moser, 1998). Perpendicular to the transverse axis and major HF subfields (DG, CA, etc.), the longitudinal axis of the rodent HF runs from the septal nuclei rostrally to the temporal cortex caudally, and is thus often known as the septo-temporal axis (Amaral *et al.*, 2007). A multitude of research points to a functional gradient across this axis of the mammalian HF (Strange *et al.*,

2014), which can accordingly be segmented into three distinct regions. Specifically, an intermediate zone with overlapping functions separates the septal pole, known as the dorsal hippocampus in rodents and the posterior hippocampus in primates, from the temporal pole, or the ventral rodent/anterior primate hippocampus (Fanselow and Dong, 2010). In general, the intrinsic connections of the transverse loop are repeated over the length of the septo-temporal axis, with pathways between DG, CA3, CA1 and the subiculum mainly linking septal to septal, intermediate to intermediate and temporal to temporal (Fanselow and Dong, 2010). However, afferent and efferent connectivity shifts between the septal and temporal poles (Moser and Moser, 1998).

Collectively, evidence indicates that the septal region of the mammalian hippocampus is primarily involved in functions relating to spatial cognitive processing and memory (Moser and Moser, 1998; Bannerman *et al*., 1999). In terms of extrinsic connectivity, visuo-spatial information from the primary sensory and associative cortices predominantly enters the septal two-thirds of the DG (Moser and Moser, 1998). Major direct and indirect projection targets of the septal HF (emanating largely from CA1 and the subiculum) include cortical areas such as the retrosplenial and anterior cingulate cortices (Cenquizca and Swanson, 2007), which are heavily involved in the processing of visuospatial information, memory and environmental exploration (Fanselow and Dong, 2010). The septal HF also projects to subcortical regions including the medial and lateral mammillary nuclei and the anterior thalamic complex, forming a circuit thought to generate cognitive maps for navigation (Muller *et al.*, 1996; Fanselow and Dong, 2010), and the ventral tegmental area and reticular part of the substantial nigra, crucial for locomotion (Swanson and Kalivas, 2000) and the orienting of movements (Hikosaka and Wurtz, 1983) respectively. These latter systems are key drivers of exploratory or foraging behaviour (Swanson and Kalivas, 2000; Fanselow and Dong, 2010).

Accordingly, gene expression in the septal hippocampus correlates with that in cortical information processing regions (Fanselow and Dong, 2010). Lesions specific to the septal rat hippocampus produce preferential deficits in spatial memory formation during water (Moser *et al.*, 1995) and radial arm (Pothuizen *et al.*, 2004) maze tasks. In monkeys, a higher proportion of neurons are active in the septal than the temporal hippocampus during the delay period of a spatial delayed matching-to-sample task (Colombo *et al.*, 1998), while functional imaging demonstrates greater activation of the septal human HF during the encoding of various modalities of memory, including word lists, visual associations and novel pictures (Moser and Moser, 1998).

Meanwhile, the temporal hippocampal region has qualitatively different functions (Moser and Moser, 1998) which relate predominantly to stress and affective modulation (Sahay and Hen, 2007; Fanselow and Dong, 2010). Unlike the septal HF, the temporal HF projects directly to the olfactory bulb and other primary olfactory areas of the cortex (Cenquizca and Swanson, 2007). The temporal HF has extensive bi-directional connections with multiple amygdalar nuclei (Fanselow and Dong, 2010) and with the infralimbic, prelimbic, and agranular insular cortices (Chiba, 2000). These structures exhibit parallel descending projections (via the lateral septum, medial and central amygdalar nuclei, and bed nuclei of the stria terminalis (BNST)) to innervate the periventricular and medial zones of the hypothalamus (Herman *et al.*, 2005; Fanselow and Dong, 2010). The hypothalamus is the primary coordinator of autonomic, neuroendocrine and motor responses during emotionally motivated behaviours, relating to defence, reproduction and ingestion (Petrovich *et al.*, 2001). The BNST acts as a crucial relay station for hippocampal regulation of the HPA response to psychological stress (Herman *et al.*, 2005), and its anteromedial nuclei extend rare direct projections to inhibit HPA-effector (CRF) neurons in the PVN (Cullinan *et al.*, 1993; Fanselow and Dong, 2010). Though retrograde tracing has also pointed to a direct projection from the temporal subiculum to the PVN, this was not confirmed through anterograde tracing (Jacobson and Sapolsky, 1991). Nevertheless, several other targets of excitatory output neurons of the temporal subiculum are known to have inhibitory influences on the PVN, including the peri and sub-paraventricular hypothalamus, the ventrolateral preoptic area, and the ventrolateral dorsomedial hypothalamus (Smulders, 2017). Additionally, the temporal HF projects directly to the central amygdalar nucleus, which may mediate hippocampal contributions to affective aspects of fear learning (Fanselow and Dong, 2010), and the caudomedial (shell) region of the nucleus accumbens, which is central to the processing of reward and motivation of feeding behaviour (Wassum *et al.*, 2009).

Gene expression in the temporal hippocampus mirrors that in the amygdala and hypothalamus (Fanselow and Dong, 2010), supporting a similar role in the modulation of emotion and stress. Lesions of the temporal subiculum enhance CORT secretion following psychogenic stressors, thus interfering with recovery from HPA-axis activation (Herman and Mueller, 2006). Furthermore, lesions to the temporal hippocampus alone exacerbate the development of gastric ulcers driven by cold or restraint stress in rats (Henke, 1990). Damage to the temporal-most quarter of the HF also reduces the expression of unconditioned fear behaviours, including avoidance of open maze arms, and dampens the CORT response to confinement in a brightly lit space (Kjelstrup *et al.*, 2002). These effects were not replicated by damage to the septal three-quarters of the HF or to the amygdala, while temporal lesions did not impair contextual fear

conditioning or spatial navigation. However, these functional dissociations are not absolute, with some studies pointing to a role of the temporal hippocampus in spatial memory and the context-processing component of fearing conditioning (Fanselow and Dong, 2010).

1.4 Adult Hippocampal Neurogenesis (AHN)

Historically, it was commonly accepted that the genesis of neurons in mammals and birds occurs only during a discrete period of embryonic development (Altman, 1962). This dogma was first challenged by Altman (1962), who created lesions in the brains of adult rats and injected a radioactive label to measure proliferation of glial cells induced by the injury. Surprisingly, in addition to the predicted glial response, the nuclei of some neurons and neuroblasts were also labelled in forebrain regions unrelated to the lesions. Adult neurons do not undergo mitotic division, and the authors concluded that the labelled neuronal cells had originated from previously undifferentiated cells dividing during the period that the radioactive substance was available.

Subsequently, autoradiographic studies in undamaged adult rats identified a proliferative region of granule cells in the DG subfield of the hippocampus (Altman, 1963; Kaplan and Hinds, 1977). Specificity of adult hippocampal neurogenesis (AHN) to granule cells of the DG was supported by an absence of both labelled polymorph cells in this region and of labelled pyramidal cells in the adjacent Ammon's horn (Altman and Das, 1965). For many years, the existence of AHN in the mammalian brain was met with continuing skepticism (e.g. Rakic, 1985). Nevertheless, post-natal hippocampal neurogenesis was also demonstrated in guinea-pigs (Altman and Das, 1967), rabbits (Guéneau *et al.*, 1982), cats (Altman, 1963) and tree shrews (Gould *et al.*, 1997). The prevailing view eventually changed (Gross, 2000) as the genesis of new neurons was confirmed to occur in the adult primate brain; within the hippocampus of marmoset monkeys (Gould *et al.*, 1998) and humans (Eriksson *et al.*, 1998). A region known as the subventricular zone, which lines the wall of the lateral ventricles, is also neurogenic during adulthood (Kuhn *et al.*, 1996) and gives rise to neuroblasts that migrate through the rostral migratory stream to reach their final position in the olfactory bulb (Melleu *et al.*, 2016). Neurogenesis in this region is sometimes measured as a control for the specificity of particular environmental influences on AHN.

1.4.1 Stages of AHN

The development of new neurons through AHN consists of multiple stages, which can be summarised as follows: *i)* proliferation, or the birth of new cells; *ii)* migration and neuronal

differentiation; *iii)* maturation/survival of immature neurons; and *iv)* functional integration of new mature neurons into the pre-existing neural circuitry (Jorgensen *et al.*, 2019). As neurons of all developmental stages can be found at any one time point, AHN is not a population event resembling the sequential progression of embryonic neurogenesis, but is instead an individualized process (Kempermann *et al.*, 2004). Certain environmental factors mediate AHN, and these influences may be dissociated between the various stages (e.g. Steiner *et al.*, 2008). For this reason, the specific morphological and temporal characteristics of each phase are described in more detail below, according to studies conducted in rats and mice. The various methods for quantifying AHN that were employed to delineate these stages are detailed in section 1.4.2.

AHN begins with a population of neural stem cells residing in the neurogenic niche of the subgranular zone (SGZ), which lines the granule cell layer of the DG. Quiescent neural progenitors (QNPs, also known as neural precursors, radial astrocytes and type-1 cells) resemble radial-glia, with triangular soma and a long apical process extending from the SGZ to the molecular layer (Encinas *et al.*, 2011). They also share electrophysiological characteristics with astroglia (Filippov *et al.*, 2003), and express the astrocytic marker glial fibrillary acidic protein (GFAP). However, QNPs are distinct from mature hippocampal astrocytes in their expression of the intermediate filament nestin, and are unique in their ability to produce neurons (Encinas *et al.*, 2011). QNPs make up around two-thirds of nestin-expressing cells in the adult mouse SGZ (Kempermann *et al.*, 2004), but their population displays a relatively low level of proliferation (Encinas *et al.*, 2011), accounting for only 5% of divisions by nestin-positive cells in this area (Kronenberg *et al.*, 2003). QNPs are bipotent and, upon activation, undergo a rapid series of asymmetric divisions which produce intermediate stem cells known as amplifying neural progenitors (ANPs) (Encinas *et al.*, 2011). Following an average of three divisions, QNPs begin differentiation into newly-born mature astrocytes and thus exit the progenitor pool, leading to declining numbers of remaining hippocampal stem cells with age (Kuhn *et al.*, 1996; Olariu *et al.*, 2007; Encinas *et al.*, 2011).

The ANP daughter cells (or type-2 cells) also express nestin, but lack astrocytic features, including expression of GFAP. In terms of morphology, type-2 cells are round or oval in shape with a dense, irregularly shaped nucleus and short, tangential processes. ANPs re-enter the cell cycle and exhibit a high level of transient proliferation, accounting for the majority of divisions by nestin-expressing cells in the SGZ (Filippov *et al.*, 2003; Kempermann *et al.*, 2004). Amplifying type-2 progenitors are sub- classified according to whether they express the early neuronal marker doublecortin (DCX): a feature that is absent in type-2a cells but present in

type-2b. Interestingly, immunoreactivity for Prox-1, a transcription factor specific to hippocampal granule neurons (Pleasure *et al.*, 2000), is also first detectable in type-2b cells, suggesting early determination of neuronal fate (Kronenberg *et al.*, 2003). While around 80% of proliferating DG cells eventually differentiate into mature neurons, approximately 15% become astroglia (Lee *et al.*, 2006).

Concomitant with DCX expression, ANPs begin to migrate the short distance from their site of division in the SGZ into the overlaying granule cell layer of the DG (Kuhn *et al.*, 1996). Active migration requires cytoskeletal changes, as neuroblasts extend a leading process toward their target destination, before translocation of the cell body (Francis *et al.*, 1999). DCX plays a role in regulating microtubule-based vesicle transport, which is critical to neuronal migration and the outgrowth of axons (Deuel *et al.*, 2006). DCX is also a marker of neuronal differentiation (Francis *et al.*, 1999), with expression associated with changes in morphology and structural plasticity (Klempin *et al.*, 2001). As cells transition to type-3, DCX expression is maintained alongside active division, as indicated by persistence of proliferative marker Ki-67 (Kronenberg *et al.*, 2003). Unlike earlier progenitors, type-3 cells do not express nestin, and also exhibit notable morphological changes, including a rounded nucleus (Kempermann *et al.*, 2004). Virtually all DCX^+ progenitor cells also express polysialated neural cell adhesion molecule (PSA-NCAM) (Kronenberg *et al.*, 2003), the embryonic form of neural cell adhesion molecule (Seki and Arai, 1993).

Expression of DCX continues as immature granule cells become post-mitotic, in a phase characterised by the transient co-expression of the calcium-binding protein calretinin, which begins one or two days after cell cycle exit (Brandt *et al.*, 2003). Just three days after the initial cell division, two-thirds of new-born cells have been reported to already express post-mitotic markers (Kempermann *et al.*, 2004), while the transition from ANPs to post-mitotic neuroblasts is virtually complete after five to seven days (Encinas *et al.*, 2011). By this time, proliferation has resulted in a four- or five-fold expansion in the total population of new cells (Kempermann *et al.*, 2004), with numbers peaking around one week (Gould *et al.*, 1999). More than 70% of the population of DCX^+ cells in the adult mouse DG are estimated to be post-mitotic (Plümpe *et al.*, 2006), and DCX-expression is highest during the second week of neuronal development (Couillard-Despres *et al.*, 2005). As neuronal differentiation continues, immature neuroblasts (typically found at the base of the granule cell layer), extend dendrites towards the adjacent molecular layer (Kuhn *et al.*, 1996; Ihunwo *et al.*, 2016). Immature neurons also project axons through the hilus and towards area CA3, which they first reach after 11 days (Zhao *et al.*, 2006). Over approximately four days following the cessation of mitosis, there is a period of massive

cell death, wherein a majority of the population of immature neurons is eliminated through large scale apoptosis (Gould *et al.*, 1999; Kempermann *et al.*, 2004). Thus, while expansion occurs at the level of precursor cells, long lasting survival is determined during the early post-mitotic period, prior to terminal differentiation.

Increasing maturation is accompanied by expression of mature neuronal marker NeuN (Cameron *et al.*, 1993). Overlap between NeuN and DCX expression has been observed between 10 and 14 days (Brown *et al.*, 2003b), consistent with the differentiated granule cell phenotype exhibited by a significant number of DCX^+ cells in the adult rat DG (Rao and Shetty, 2004). DCX expression is subsequently downregulated (Couillard-Despres *et al.*, 2005), to be detectable in only 2% of BrdU-labelled cells after one month, and in no cells after two (Brown *et al.*, 2003b). Calretinin expression also overlaps transiently with the expression of NeuN (Brandt *et al.*, 2003). Over a period of around three weeks, dendritic arborisation in the molecular layer further develops (Braun and Jessberger, 2014), spine growth peaks, axons extend further into CA3 (Zhao *et al.*, 2006) and synapses are formed with hilar interneurons, mossy cells and CA3 pyramidal cells (Toni *et al.*, 2008). After three to four weeks, NeuN is expressed by more than 70 to 80% of young cells (Cameron *et al.*, 1993; Brown *et al.*, 2003b).

Evidence suggests that, following loss in the early post-mitotic phase, levels of immature cells stabilise around a month after the initial division, and remain consistent for at least eleven months subsequently (Kempermann *et al.*, 2003). Therefore, cells that survive for four weeks are likely to be present a year later. After four weeks, 60 to 70% of new-born neurons express calbindin (Kempermann *et al.*, 1997a), which replaces calretinin in mature granule cells (Brandt *et al.*, 2003). One month old neurons extend axonal connections to the mossy-fibre layer of DG and are indistinguishable in their projections from neighbouring granule cells (Stanfield and Trice, 1988; Cameron *et al.*, 1993). Adult-generated neurons also have electrophysiological properties comparable to mature granule cells (Mongiat and Schinder, 2011) and appear to be functionally integrated into the existing neuronal network (van Praag *et al.*, 2002; Overstreet *et al.*, 2004; Schmidt-Hieber *et al.*, 2004). For example, adult-born neurons participate similarly in immediate early gene expression during acquisition of learning tasks, suggesting their contribution to hippocampal-dependent functions (Jessberger and Kempermann, 2003).

1.4.2 Methods for quantifying AHN

Early studies of AHN quantified proliferation in the central nervous system using *in vivo* injection of thymidine, a specific precursor of chromosomal DNA. Thymidine is also known as DNA nucleoside T, and pairs with deoxyadenosine (nucleoside A) in double-stranded DNA,

meaning it is incorporated into the DNA of dividing cells during the S phase of mitosis (Altman, 1962). Titriated thymidine (or thymidine-^{3}H) is detectable due to its inclusion of the hydrogen α-emitting radionuclide, tritium. Nuclear labelling with thymidine-^{3}H occurs during the 60 minutes after administration, while the substance is largely degraded within 60–90 minutes (Alvarez-Buylla *et al.*, 1990). Radioactive nuclei of cells incorporating thymidine-^{3}H cause blackening of overlying silver grains, detectable via (fine-resolution) autoradiography (Altman and Das, 1967).

In more recent explorations, an analogue to thymidine known as bromodeoxyuridine (BrdU) has been similarly utilised for *in vivo* labelling of dividing cells during S phase, and detected in their progeny using immunohistochemistry (IHC) (del Rio and Soriano, 1989). Both of these methods effectively "birth-mark" dividing cells (Kempermann *et al.*, 2010) and can be used to track their development, movement and fate, while indicating relative cell survival. However, these compounds have certain limitations as neurogenic markers. Notably, *in vivo* administration precludes most studies in humans, while toxicity at higher doses means BrdU is often given at a sub-saturation threshold, meaning that some dividing cells are not labelled. Moreover, the nuclear content of the markers is diluted with each subsequent division of an incorporating cell. Concern has also been raised that treatments affecting rates of proliferation may also influence the bioavailability of thymidine or BrdU (Couillard-Despres *et al.*, 2005), for example by systemically altering permeability of the blood-brain barrier (Wu *et al.*, 2014), though many studies include controls to discount this possibility.

An alternative method of quantifying AHN involves post-mortem measurement of the endogenous proteins transiently expressed during neuronal development (Brown *et al.*, 2003b) using IHC. Levels of proliferation are reflected by markers of mitosis, such as proliferating cell nuclear antigen (PCNA), Ki-67 and minichromosome maintenance protein 2 (MCM2) (Lucassen *et al.*, 2010), while neuronal migration and differentiation are reflected by expression of DCX, PSA-NCAM and calretinin. Finally, common markers characteristic of mature neurons include NeuN, calbindin and neuron specific enolase. IHC double labelling can also be used to measure co-expression of BrdU with these transient markers, in order to dissociate cells of differing developmental stages or to quantify cell survival after known time spans. However, DCX protein levels are detectable through IHC only in immature neurons, and not during physiological or reactive gliogenesis (Couillard-Despres *et al.*, 2005), which means that double-labelling is not necessary in order to establish neuronal identity (LaDage *et al.*, 2010a). Moreover, staining for DCX alone has been reported to produce similar estimates of cell

numbers to BrdU labelling (Rao and Shetty, 2004), including with respect to changes in AHN rates that occur in response to behavioural experiences (Couillard-Despres *et al.*, 2005).

1.5 AHN Co-varies with Long-Term Experience in a Valence-Specific Direction

Under non-manipulated conditions, differences in rates of AHN can be observed across the septo-temporal axis of the rodent DG. While numbers of DCX^+ immature neurons are reported to be higher in the septal subregion (Snyder *et al.*, 2009b; Tanti *et al.*, 2013), numbers of incorporated adult-born mature neurons are greater in the temporal DG (Tanti *et al.*, 2012; Tanti *et al.*, 2013; Anacker and Hen, 2017). Moreover, AHN levels in the mammalian hippocampus are regulated by a variety of long-term experiences, and relative sensitivity to particular conditions across the septo-temporal axis may relate to the subregional specialisations detailed in section 1.3.2. Experience-driven alterations in proliferative activity may reflect a change in either the number of dividing cells, the rate of cell division, or a combination of both. Certain conditions or experiences may further influence neuronal fate determination by altering the proportion of precursor cells that differentiate into neurons, as opposed to other (glial) cell types, in addition to modulating the number or proportion of immature neurons that survive to later stages of maturity. As the population of cells expressing immature neuronal markers (such as DCX) is much larger than the population of mature neurons that survive to be integrated, the former group may represent a pool from which, under conditions that stimulate AHN, new cells can be recruited for neuronal development (Brandt *et al.*, 2003). Generally, the influence of an experience on AHN depends upon its valence, as chronic stress suppresses the proliferation and survival of new neurons, whereas experiences associated with positive affect have a converse, stimulatory effect. The following section shall review the evidence for environmental modulation of AHN in primates and rodents.

1.5.1 Evidence in humans

In humans, linguistic self-report is considered the gold-standard for characterising emotional states (Mendl *et al.*, 2010). When seeking proxy measures for conscious subjective experience, biomarkers that correlate with the valence of self-reported human mood in a directional manner are promising candidates. The primary rationale for applying indices of AHN to the assessment of animal welfare stems from observations that the process is suppressed in cases of major depressive disorder (MDD), for which chronic stress is a significant risk factor (McEwen, 2005; Levone and Cryan, 2015), but stimulated by antidepressant (AD) treatment (Jacobs *et al.*, 2000; Miller and Hen, 2015), which also improves mood.

To date, no studies have yet employed methods of birth-marking new neurons, such as BrdU labelling, in patients with MDD or anxiety disorders (Miller and Hen, 2015). A few studies have quantified proliferating cells in the DG of post-mortem brains from individuals with untreated MDD, using IHC. Levels of MCM2-labelled progenitors were found to be lower in elderly patients with MDD than in age-matched controls (Lucassen *et al.*, 2010). However, levels of alternative mitotic marker Ki-67 showed only a trend towards suppression with MDD in one study (Boldrini *et al.*, 2009), and did not differ between patient and control groups in another (Reif *et al.*, 2006). It has been suggested that this disparity might relate to the short half-life of Ki-67 and its rarity in the human brain (Aniol and Gulyaeva, 2015).

Consistent with observations of reduced DG volume (e.g. Huang *et al.*, 2013), post-mortem analysis indicates that un-medicated patients with MDD have fewer granule neurons in the temporal- and mid-DG than control individuals, but not in the septal region (Boldrini *et al.*, 2013). Moreover, age of MDD onset correlated with cell numbers in temporal DG alone, so that duration of the disease negatively predicted granule neuron counts in this subregion. Measurement of hippocampal grey matter volume in live MDD patients using magnetic resonance imaging (MRI) has further revealed that those in the midst of a depressive episode have a smaller hippocampus than MDD patients who are in remission (Arnone *et al.*, 2013). This is consistent with an impairment in adult structural plasticity, rather than a predisposing developmental effect (Miller and Hen, 2015). If the inconsistent findings concerning levels of proliferation are not considered to be purely methodological, it has been concluded that lower DG volumes and numbers of granule cells in patients with untreated MDD must be caused by the inhibition of later stages of AHN, such as neuronal differentiation and survival (Miller and Hen, 2015).

Treatment of mood disorders with AD agents is associated with improvements in self-reported affective state (e.g.Wheatley *et al.*, 1998; Cassano *et al.*, 2002). Post-mortem studies have consistently found a greater number of granule cells and increased volume of the DG in patients taking these medications compared to un-medicated individuals (Miller and Hen, 2015). Patients receiving AD treatment for a mood disorder (MDD or bipolar disorder) had more granule neurons than untreated patients in the temporal and middle regions of the DG, and displayed granule neuron numbers comparable to individuals free from psychopathology over all dentate subregions (Boldrini *et al.*, 2014). Patients with selective serotonin reuptake inhibitor (SSRI)-treated MDD similarly exhibited more granule neurons in the mid-DG region than individuals with un-medicated MDD and had larger volumes of the temporal and middle GCL (Boldrini *et al.*, 2013). Neither study observed between group differences in the septal DG.

Longitudinal MRI studies indicate growth of the hippocampus as a whole following treatment with SSRIs (Vermetten *et al.*, 2003; Arnone *et al.*, 2013). Furthermore, high resolution MRI facilitating measurement at the level of subfields has demonstrated that, in restoring total hippocampal volume, AD treatment increases DG volume to the level of healthy control subjects, but has less impact on CA1-3, which exhibited volumes intermediate to the un-medicated and control groups, but differing from neither (Huang *et al.*, 2013).

Post-mortem reports on the proliferation of progenitor cells following AD treatment are again inconsistent (Miller and Hen, 2015). The number of Ki-67 labelled mitotic cells in treated patients with MDD was around three times greater than that in control subjects, and five times greater than that in un-treated MDD patients, with this effect largely restricted to the temporal DG (Boldrini *et al.*, 2009). However, though patients receiving both tricyclic ADs and SSRIs had more nestin-immunoreactive progenitor cells, an increase in Ki-67 labelling was largely attributable to the tricyclic class, and SSRI-treated patients had a comparable number of these cells to control and un-treated patients. A similar study confirmed that patients receiving AD treatment had more nestin-positive neural progenitors in the temporal and mid-DG than both controls and untreated patients, and more Ki-67-positive mitotic cells in the temporal DG than un-treated patients (Boldrini *et al.*, 2014). However, this study collated seven patients with MDD and three with bipolar disorder, whilst not explicitly differentiating between AD classes. In contrast, expression of MCM2 in elderly patients receiving AD treatment for MDD remained lower than for age-matched controls (Lucassen *et al.*, 2010), though this apparent lack of clinical efficacy may relate to the more advanced age of the sample. Ki-67 expression was also found not to differ for younger medicated versus un-medicated patients with bipolar disorder or MDD, but medication adherence was not confirmed and an insufficient sample of three un-medicated MDD patients precluded analysis in this subgroup alone (Reif *et al.*, 2006). Finally, Epp *et al.* (2013) reported a trend towards an increase in neuronal differentiation, reflected by the ratio of DCX- to NeuN-expressing cells, in MDD patients compared to controls. Though their analysis did not account for AD treatment in the MDD group, 10/12 of these individuals were taking some form of AD compound. A stimulating effect of this treatment is thus likely to explain this observation (Aniol and Gulyaeva, 2015).

Overall, though there is little direct evidence in humans for the influence of AD treatment on expression of DCX or other markers of neuronal differentiation and maturation, the observed increases in numbers of mature granule neurons and hippocampal volumes mediated by these compounds must be explicable in terms of an augmentation of AHN translated beyond the proliferation stage (Miller and Hen, 2015). In conjunction with studies relating impaired AHN

to cases of MDD, the literature in humans points to a directional relationship between subjective long-term mood and AHN levels. Furthermore, cancer therapies induce non-specific apoptosis of dividing cells, and the prevalent co-morbidity of depression and deficits in hippocampal-dependent memory in patients undergoing these treatments may suggest a causal role for AHN in the regulation of mood (Pereira Dias *et al.*, 2014; Miller and Hen, 2015). However, manipulations to experimentally block or ablate AHN are necessary to demonstrate causation. These explorations have been made using animal models and are discussed in section 1.5.5.

1.5.2 AHN in rats, mice & non-human primates is suppressed by negative experiences

Enduring affective states are difficult to assess objectively in non-verbal animals. However, neuroscientists have developed several behavioural tests to measure mood in laboratory rodents, and these have been validated according to the specific pharmacological effects of compounds with clinical efficacy in treating human mood disorders (Poirier *et al.*, 2019). Typical assessments of anxiety-like state in rats and mice include behaviour in an elevated plus maze (EPM), light/dark box, or open field, and the suppression of feeding in novel environments (novelty-suppressed feeding, or NSF). Anxiolytic agents consistently influence behaviour in these tests, for example increasing exploration of an open field and the open arms of an EPM, and reversing decrements in NSF (Christmas and Maxwell, 1970; Pellow *et al.*, 1985; Pellow and File, 1986; Merali *et al.*, 2003). Depression-like states are commonly measured according to indices of anhedonia (generally attenuated sucrose consumption), social avoidance, and immobility during the forced swim test (FST) and tail suspension test (TST). AD compounds reliably increase such motivated (Dulawa *et al.*, 2004; Wallace-Boone *et al.*, 2008) and hedonic (Rygula *et al.*, 2008) behaviours. Conversely, manipulations with behavioural effects opposite to these therapeutic compounds provide models of anxiety- and depression-like states.

Exposure to various chronic stressors, including (unpredictable) chronic mild stress ((U)CMS), repeated restraint and social defeat (Levone and Cryan, 2015), reliably induce depressive and anxious behaviours (e.g. Jayatissa *et al.*, 2006; Elizalde *et al.*, 2010). Moreover, these experiences are associated with notable impairments in AHN in the rodent DG (Miller and Hen, 2015), without affecting neurogenesis in the subventricular zone of lateral ventricle (e.g. Malberg and Duman, 2003).While a single instance of stress is sufficient to produce a transient suppression in the proliferation and one-week survival of hippocampal progenitors (Gould *et al.*, 1997; Tanapat *et al.*, 2001), chronic or repeated exposure to stressors often results in more sustained deficits in AHN.

Numbers of proliferating progenitor cells in the DG are suppressed by UCMS (Surget *et al*., 2011), repeated restraint (Pham *et al*., 2003; Luo *et al*., 2005; Rosenbrock *et al*., 2005; Castilla-Ortega *et al*., 2014) and psychosocial stress (Czéh *et al*., 2001; Czéh *et al*., 2002; Simon *et al*., 2005). One study observed fewer proliferating cells in the SGZ immediately after 10 days of social defeat, but found that numbers normalised over the subsequent 24-hour window (Lagace et al., 2010). In contrast, other studies have demonstrated sustained suppression of proliferation nine days (Malberg and Duman, 2003) or three to four weeks after stress was terminated (Luo *et al*., 2005; Elizalde *et al*., 2010). Studies exploring a potential septo-temporal gradient in the sensitivity of hippocampal proliferation have yielded mixed results. Variously, stress-induced reductions in proliferation have been observed only in the temporal DG (Jayatissa *et al*., 2006; Nollet *et al*., 2012), in both the septal and temporal DG, but with a greater magnitude in the latter (Hawley and Leasure, 2012; Nollet *et al*., 2012), or to occur homogenously across the septo-temporal axis (Gould *et al*., 1998; Malberg and Duman, 2003; Luo *et al*., 2005; Elizalde *et al*., 2010; Perera *et al*., 2011; Surget *et al*., 2011; Tanti *et al*., 2013). Other studies have found no influence of various stress regimes on the proliferation stage (Lee *et al*., 2006; Hanson *et al*., 2011; O'Leary *et al*., 2012).

In terms of neuronal differentiation, repeated restraint reduced numbers of DCX^+ cells with mature neuronal morphologies (Castilla-Ortega *et al.*, 2014). A sustained effect was suggested by the finding that DCX^+ cell numbers were still reduced at the end of a 3-month individual housing period that followed a short (5-day) period of resident-intruder defeat (Van Bokhoven *et al.*, 2011). This reduction was also more pronounced for DCX^+ cells with relatively mature dendritic morphology. As control rats were housed in pairs throughout, the relative contribution of the social defeat paradigm and subsequent individual housing cannot be distinguished, though one month of social isolation alone is a stressor capable of suppressing DCX^+ cell levels (Dranovsky *et al.*, 2011). Certain studies have found reductions in DCX^+ cell numbers to be restricted to the temporal DG (Perera *et al*., 2011; Tanti *et al*., 2013), or to be significantly greater in this region than in the septal DG (Hawley *et al.*, 2012). Other explorations observed similar effects of stress across the septo-temporal axis (Nollet *et al*., 2012; de Andrade *et al*., 2013; Wu *et al*., 2014). Conversely, chronic restraint stress increased the number of cells immunoreactive for migratory marker PSA-NCAM in the SGZ, though this finding was attributed to an increase in the amount of PSA attached to NCAM molecules in pre-existing granule neurons, rather than a change in AHN (Pham *et al.*, 2003). Repeated application of restraint for five minutes at the same time each day also unexpectedly increased DCX^+ cell numbers in the SGZ-GCL three days later, in conjunction with attenuated anxious and

depressive behaviour and improved memory retention (Parihar *et al.*, 2011). It may be that the predictability and short duration of the restraint protocol constituted a form of "stress inoculation", which has positive consequences for coping (Brockhurst *et al.*, 2015). Importantly, the directional relationship between AHN and the valence of the associated (behaviourally indicated) affective state was maintained.

The survival of immature neurons has been shown to be down-regulated preferentially in the temporal DG by stress (Elizalde *et al.*, 2010; Hawley and Leasure, 2012; Tanti *et al.*, 2012), and this region-specific effect persisted for the following intervention-free month (Elizalde *et al.*, 2010; Tanti *et al.*, 2012). However, long-term survival after labelling during the successive month was no longer affected (Elizalde *et al.*, 2010). Another study found that immature neuronal survival was decreased over the whole hippocampus, though the mean suppression was greater in the temporal region (Nollet *et al.*, 2012). In other cases, survival was attenuated in both the septal and temporal DG (Surget *et al.*, 2011; Hawley *et al.*, 2012), and this impairment continued for the month after stress had ceased (Surget *et al.*, 2011). In contrast, eight week survival of $BrdU^+$ cells was not influenced by ongoing separation stress in bonnet monkeys (Perera *et al.*, 2011), and one or three week survival was not altered by multiple stress paradigms in adult rats (Hanson *et al.*, 2011).

Some studies have also explored how the survival of existing cells labelled prior to the commencement of chronic stress is affected by this experience. Restraint stress negatively impacted the survival of $BrdU^+$ cells labelled a week previously that co-expressed DCX (Castilla-Ortega *et al.*, 2014), and fewer $BrdU^+$ cells labelled prior to chronic social stress survived this experience (Czéh *et al.*, 2002). For studies exploring a septo-temporal gradient, the four-week survival of cells labelled two weeks before continued social defeat was substantially decreased in the temporal DG alone, with no effect in the septal region (Lehmann *et al.*, 2013). However, the survival of pre-existing $BrdU^+$ cells (labelled the day prior to commencement of the procedure) was comparably reduced in the septal and temporal GCL by 19 days of CMS (Lee *et al.*, 2006). Other studies observed no effect of subsequent stress on the survival of pre-labelled $BrdU^+$ cells across the septo-temporal axis (Pham *et al.*, 2003; Lagace *et al.*, 2010; O'Leary *et al.*, 2012).

Administration of exogenous CORT mirrors the effects of induced psychological stress on AHN (Cameron and Gould, 1994; Miller and Hen, 2015), with a high dose suppressing Ki-67 proliferating cells and DCX^+ cell densities in the whole DG of male rats, and the temporal DG of females (Brummelte and Galea, 2010). Moreover, adrenalectomy indicates that the

suppression of AHN by social defeat is at least partially mediated by CORT secreted during this experience, as adrenalectomised mice show enhanced cell survival regardless of stressful experience (Lehmann *et al.*, 2013). Impaired AHN is also observed in models of depression that are not caused by chronic stress (Miller and Hen, 2015). For example, rodent models of hypothyroidism, which leads to depression in humans, are characterised by both depressive-like behaviours and reduced AHN (Montero-Pedrazuela *et al.*, 2006). The chronic experience of pain is also associated with the induction of anxiety- and depressive-like behaviours and the suppression of AHN (Duric and McCarson, 2006; Mutso *et al.*, 2012; Dellarole *et al.*, 2014; Dimitrov *et al.*, 2014; Romero-Grimaldi *et al.*, 2015). As such, there is substantial evidence to support the conclusion that AHN in rodents co-varies with behavioural indicators of negative affective state and is suppressed by various factors associated with low mood in humans. Some studies suggest that AHN in the temporal hippocampus is especially vulnerable to chronic stress, though others report homogenous effects across the septo-temporal axis. Given that the repeated experience of psychological stress from multiple sources acts to downregulate AHN, the process may have utility as a biomarker of low welfare in farmed animals. Moreover, relatively high levels of AHN may also reflect positive experiences.

1.5.3 AHN in rodents is stimulated by long term positive experience

Generally, levels of AHN in rats and mice are increased by interventions that are associated with beneficial effects for cognition and mood, including AD treatment, exercise, environmental enrichment and learning (Sahay *et al.*, 2011). A few studies have also explored the influence of AD treatment on AHN in non-human primates. In the following section, the relationship between these positive experiences and the modulation of various stages of neuronal development will be explored in further detail.

1.5.3.1 Antidepressant treatment

Latencies to feed in a novel environment reflect anxiety in mice, and are reduced by chronic AD treatment even under non-stress conditions (Santarelli *et al.*, 2003). According to a similar time course, chronic (lasting ≥ 11 days), but not acute, treatment with several ADs compounds has a stimulatory effect on AHN, with no influence on neurogenesis in the subventricular zone of the lateral ventricle (Malberg, 2000; Manev *et al*., 2001; Santarelli *et al.*, 2003; Banasr *et al.*, 2006; Encinas *et al*., 2006). Interestingly, the effect of fluoxetine on proliferation is dissociable between classes of neural precursor, as this treatment increased the number of $BrdU^+$ ANP daughter cells and their rate of symmetric divisions, without influencing numbers of GFAP-expressing QNPs or their asymmetric division rate (Encinas *et al.*, 2006). A treatment analogous

to electroconvulsive therapy (an effective short-term AD in humans) similarly increased proliferation in the DG of adult bonnet monkeys immediately post-intervention, in an effect that was sustained four weeks later (Perera *et al.*, 2007).

In unstressed mice, chronic fluoxetine treatment increased numbers of differentiating PSA-NCAM and DCX^+ cells (Encinas *et al.*, 2006), without altering the number of neuroblasts co-expressing NeuN. In a study that did not quantify cells in the septal region, fluoxetine treatment similarly upregulated numbers of $BrdU^+/DCX^+$ immature neurons in the temporal rat DG (Airan *et al.*, 2007). However, proportions of cells differentiating into neurons versus astroglia are not affected by AD treatment (Malberg, 2000; Santarelli *et al.*, 2003; Encinas *et al.*, 2006), indicating a lack of impact on neuronal fate determination. Both repeated shock therapy (Perera *et al.*, 2007) and continued AD treatment increased numbers of $BrdU^+$ new neurons surviving over a period of four weeks (Encinas *et al.*, 2006; Kong *et al.*, 2008). However, this effect appears to be transient, as one month after the discontinuation of fluoxetine treatment, numbers of proliferating ANP cells, DCX and PSA-$NCAM^+$ immature neurons (Encinas *et al.*, 2006) and $BrdU^+$ mature neurons (Malberg, 2000) did not differ in number from vehicle-treated controls.

Relatively few studies have explored differential responsivity of AHN across the septo-temporal axis to AD treatment under basal/non-stress conditions. However, non-stressed monkeys given fluoxetine had a 300% higher rate of DCX^+ cells in the temporal DG compared to the non-stressed placebo group, with no such difference present within the septal region (Perera *et al.*, 2011). Fluoxetine treatment in control mice also upregulated proliferation and survival specifically in the temporal DG (Tanti *et al.*, 2013). Three-week administration of the atypical AD agomelatine similarly increased the three-week survival of $BrdU^+$ cells specifically within the temporal DG, but continuation of treatment during the three weeks after BrdU injections (6-week total) led to increased survival in both the septal and temporal hippocampus (Banasr *et al.*, 2006). Regional specificity may thus depend on the duration of treatment. Four weeks of fluoxetine was also found to increase the survival of immature neurons in both the septal and temporal hippocampus under basal conditions (Nollet *et al.*, 2012). Other studies have found either no, or septal subregion-specific, stimulatory effects of ADs on proliferation (Jayatissa *et al.*, 2006; Perera *et al.*, 2011; Nollet *et al.*, 2012) and survival (Elizalde *et al.*, 2010; Wu *et al.*, 2014) in unstressed animals. In one instance, DCX^+ differentiating neurons were actually fewer across the whole DG of unstressed baboons given fluoxetine (Wu *et al.*, 2014). Such inconsistent findings have led to the conclusion that experimental exposure to chronic stress is required for the reliable stimulation of AHN by AD treatment (Miller and Hen, 2015).

Substantial evidence indicates that the reversal of chronic stress-induced anxiety- and depressive-like behaviours by AD agents is associated with the recovery of deficits in AHN (Miller and Hen, 2015), and these effects are often restricted to the temporal DG subregion. AD administration reversed the deficits in numbers of $BrdU^+$ proliferating cells induced by previous inescapable foot-shocks (Malberg and Duman, 2003), repeated restraint (Luo *et al.*, 2005), CMS (Kong *et al.*, 2008) and UCMS (Surget *et al.*, 2011; Tanti *et al.*, 2013), and prevented the decrease mediated by psychosocial stress occurring in non-medicated animals when given concurrently (Czéh *et al.*, 2001). In terms of subregional specificity, ADs appear to reverse or prevent stress-mediated deficits in proliferation in whichever areas were negatively affected in untreated animals: either within the temporal subregion alone (Tanti *et al.*, 2012), or across the whole DG (Elizalde *et al.*, 2010; Nollet *et al.*, 2012). In monkeys, however, the number of $Ki\text{-}67^+$ proliferating cells in the SGZ was downregulated by separation stress but unaffected by fluoxetine treatment, though this had a marked effect on later stages of AHN (Perera *et al.*, 2011).

When social defeat stress was followed by an intermediate (nine-week) period of individual housing, AD treatment completely restored DCX^+ cell numbers, to be higher than those in the untreated-stress group and comparable to pair-housed untreated-control & AD-control rats (Van Bokhoven *et al.*, 2011). Bonnet monkeys given fluoxetine during a chronic separation stress regime exhibited 250% more type-3 DCX^+ neurons in the temporal DG compared to the unstressed-placebo group, with no effect in the septal DG, while this treatment protected from the emergence of depressive behaviours (Perera *et al.*, 2011). In mice exposed to UCMS, concurrent fluoxetine treatment prevented reductions in DCX^+ cells observed in both the septal and temporal DG of un-medicated animals (Nollet *et al.*, 2012).

The influence of AD treatment on AHN has been associated with individual differences in pharmacological efficacy, in terms of reversing behavioural depressive symptoms. Jayatissa *et al.* (2006) found that four-week administration of the SSRI escitalopram during CMS reversed anhedonia (decreased sucrose intake) in around 50% of rats, termed responders. These animals had more proliferating $BrdU^+$ cells in the temporal GCL than both CMS-vehicle and CMS-non-responder rats. AHN was thus only restored in individuals showing concurrent improvements in affective state, validating its association with mood. Furthermore, in treated rats, sucrose intake was positively correlated with number of new cells in the temporal GCL. CMS-treated rats additionally had more labelled cells than CMS-vehicle rats in the septal GCL, but the number of new cells did not differ significantly between the responder and non-responder groups in this subregion, further supporting preferential sensitivity of the temporal DG.

Findings regarding the subregional-specificity of AD-effects on neuronal cell survival are mixed. One study found that concurrent fluoxetine treatment prevented the attenuation of $BrdU^+$ four-week cell survival by UCMS (Surget *et al.*, 2011), but did not dissociate the septal and temporal DG. Fluoxetine reversed both a temporal-specific reduction in the four-week survival of $BrdU^+/NeuN^+$ cells driven by UCMS (Tanti *et al.*, 2012), and a UCMS-induced deficit in the same measure that affected both parts of the DG (Nollet *et al*., 2012; Tanti *et al*., 2013). While a five-week AD (paroxetine) course beginning during CMS normalised sucrose intake and restored proliferation compared to vehicle-controls (Elizalde *et al.*, 2010), the treatment had no therapeutic effect on the one-month survival of new cells in either DG subregion. Lithium, a compound used to treat bipolar disorder, significantly increased the number of proliferating $BrdU^+$ cells in the temporal SGZ of stressed, but not non-stressed mice, but reduced the 21-day survival of newly-born cells in both stressed and non-stressed mice. This impairment influenced the septal hippocampus of both stressed and non-stressed mice, and the temporal hippocampus of stressed mice only (O'Leary *et al.*, 2012).

Discrepancies between studies may relate to methodology or the specific actions of particular pharmacological compounds, but generally support a stimulatory influence of AD treatment on the proliferation, neuronal differentiation and survival of new neurons under basal and stress-conditions, which is associated with reduced anxious and depressive behaviour. The temporal subregion of the DG is preferentially implicated in sensitivity to stress and the AD response in some instances, though several experiences and treatments affect AHN across the length of the structure.

1.5.3.2 Exercise

Running exercise has antidepressant and anxiolytic behavioural effects in mice, resulting in decreased learned helplessness, less immobility during the FST and TST, greater durations of time spent in the open arms of an EPM (Duman *et al.*, 2008), attenuated acoustic startle and stress-induced hypothermia responses, and greater social interaction (Salam *et al.*, 2009). These positive effects occur in spite of the fact that physical exercise stimulates the HPA-axis and elevates CORT levels in rodents, both acutely during the activity and at peak periods of the circadian cycle (Stranahan *et al*., 2006; Droste *et al*., 2008; Lehmann *et al*., 2013). On the other hand, exercise robustly upregulates AHN, without influencing either proliferation in the subventricular zone of the lateral ventricle or the survival of subsequently differentiated neurons in the olfactory bulb four weeks later (Brown *et al.*, 2003a). This presents a strong example of

the valence-specific nature of the relationship between AHN and experience, as compared to CORT release, which simply indicates arousal.

When housed with free access to a running wheel, mice begin to run spontaneously on day one, and distances travelled tend to increase during the first 10 or so days, before plateauing at relatively high levels (Garrett *et al.*, 2012), typically ranging from around four to 20 km per day (reviewed in Manzanares *et al.*, 2019). Just 24-hours of housing with a running wheel proved sufficient to increase proliferating $BrdU^+$ cell numbers in the mouse DG (Steiner *et al.*, 2008), while a similar effect has consistently been observed following exercise periods ranging from seven to 14 days (Van Praag *et al.*, 1999b; Brown *et al.*, 2003a; Jin *et al.*, 2008; Kannangara *et al.*, 2009), including in rats (Redila and Christie, 2006). Such running durations similarly upregulate numbers of proliferating cells expressing PCNA (Snyder *et al.*, 2009a) (Kannangara *et al.*, 2009) and Ki-67 (Jin *et al.*, 2008). Moreover, expression of neuronal differentiation markers DCX and calretinin was also increased when compared to sedentary controls (Jin *et al.*, 2008; Kannangara *et al.*, 2009; Nokia *et al.*, 2016). Four to five weeks of regular running on a treadmill also increases proliferation (Wu *et al*., 2007; Kim *et al*., 2015; Kim *et al*., 2017), numbers of DCX^+ neuroblasts (Wu *et al*., 2007; Kim *et al*., 2017) and survival of immature neurons (Kim *et al*., 2015; Kim *et al*., 2015) in the DG of rats and mice.

There is evidence to suggest that the pro-proliferative effect of running is transient. $BrdU^+$ cell proliferation has been reported to peak after three days of running and return to baseline after 32 days (Kronenberg *et al.*, 2006). Likewise, while $PCNA^+$ cell counts were higher in running mice than sedentary controls 12 days after exercise began, this relative increase was no longer apparent 19 days after the introduction of running wheels (Snyder *et al.*, 2009a). A further study also observed no effect of 22 days of exercise on PCNA expression (Castilla-Ortega *et al.*, 2014). Employing Ki-67 as a marker, 14-day runners had a significantly increased number of proliferating cells in the DG compared to control mice. Though 28-day runners still showed a greater mean number of $Ki\text{-}67^+$ cells than controls, this difference was no longer significant. However, both 14- and 28-days running increased numbers of DCX^+ differentiating neurons (Garrett *et al.*, 2012), and the population of more mature DCX^+ cells, defined according to dendritic morphology, was expanded following 22 days of running (Castilla-Ortega *et al.*, 2014). It is not clear whether this effect would also have disappeared following an additional delay.

Through expanding the size of the precursor pool, stimulation of proliferation by exercise leads to a net increase in cell survival (Olson *et al.*, 2006). BrdU-injections during the final three days

of a 10-day running period increased net neurogenesis, in terms of surviving $BrdU^+/NeuN^+$ cells, when measured 35 days after this exercise was discontinued (Fabel *et al.*, 2009). This indicates that even in the absence of continued activity-dependent input during the post-mitotic phase, enhanced proliferation leads directly to a higher level of neuronal survival. As such, continued wheel running resulted in greater numbers of surviving BrdU-labelled DG cells than exhibited by control animals when measured after two (Garrett *et al.*, 2012), three (Snyder *et al.*, 2009a), four (Van Praag *et al.*, 1999b; Brandt *et al.*, 2003; Brown *et al.*, 2003a; Garrett *et al.*, 2012), eight or 16 weeks (van Praag *et al.*, 1999a), with these cells co-expressing NeuN at later time points. Furthermore, running significantly increased seizure-induced expression of intermediate early gene (Arc) in 21-day-old granule cells relative to controls, indicating that these neurons were functionally integrated (Snyder *et al.*, 2009a).

Running also enhances the survival of mitotic cells labelled before the exercise commenced. Following BrdU injections in the three days prior to exercise, the total number of surviving $BrdU^+$ cells and new-born ($BrdU^+/NeuN^+$) neurons in the DG was increased by four weeks of wheel running, either with or without a resistance load applied (Lee *et al.*, 2013). Similarly, 22 days of running increased both the survival rate of $BrdU^+$ cells labelled the day before exercise began and the total number of $BrdU^+/DCX^+$ double-labelled differentiating neurons (Castilla-Ortega *et al.*, 2014). Interestingly, individual differences in running activity linearly predict both the proliferation and survival of new hippocampal cells. In a strain of mice showing variation in running proclivity, average daily wheel rotations correlated with numbers of both proliferating $BrdU^+$ cells in the DG and those surviving 28 to 40 days post-injection (Allen *et al.*, 2001).

Not all forms of exercise have a positive influence on measures of AHN. For example, forced swimming exercise did not upregulate proliferation or the survival of immature neurons (Van Praag *et al.*, 1999b), while neither high-intensity interval treadmill training (alternating short bouts of very intense anaerobic exercise, with recovery periods), or anaerobic resistance training (climbing a vertical ladder with weights) affected AHN in adult male rats (Nokia *et al.*, 2016). The latter authors suggested that exercise must be aerobic to stimulate AHN. Generally, however, studies have conclusively demonstrated that the stimulatory influence of (wheel or treadmill) running exercise on hippocampal proliferation in rats and mice is sustained to enhance neuronal differentiation and survival, at least on a transient basis. A septo-temporal gradient in the influence of physical activity on AHN does not appear to have been investigated in isolation, but has been explored in conjunction with environmental enrichment (section 1.5.3.3).

1.5.3.3 Environmental Enrichment

Access to a running wheel often comprises one component of environmental enrichment (EE), which involves the provision of social stimulation and inanimate complexity within the home cage of rats or mice. Compared to standard housing, EE typically consists of a larger cage, housing eight to fourteen animals and containing stimuli such as toys, rearrangeable tunnels and ladders, nesting materials and a running wheel. Some protocols additionally offer food treats, or periodically alter aspects of the environment to maintain variety (Auvergne *et al.*, 2002; Veena *et al.*, 2009). Though findings are somewhat mixed, which may relate to variations between conditions and the control groups utilised (reviewed in Girbovan and Plamondon, 2013), there is evidence that EE improves behavioural indicators of mood. For example, EE-housed mice entered an open arm of the EPM sooner, and made more entries to open arms, than standard housed mice (Hendershott *et al.*, 2016), and were less immobile during the FST (Llorens-Martín *et al.*, 2007). Furthermore, EE reliably improves depressive behaviour induced by stress, for example reversing the increased immobility in the FST and decreased sucrose consumption induced by chronic restraint (Veena *et al.*, 2009). Social enrichment has clear benefits for depressive-like behaviour when compared to isolated housing, with group-housed mice exhibiting less immobility in the FST and TST (Martin and Brown, 2010). Exposure to EE also improves spatial memory ability and stimulates various aspects of neural plasticity (Brown *et al.*, 2003a), including notable enhancements in AHN.

Both exercise and EE mediate increases in net neurogenesis. However, while physical activity promotes proliferation and expands the size of the available pool of precursor cells, with a subsequent quantitative effect on neuronal survival, EE appears to have a direct preferential effect on the survival of progeny, by decreasing the normal loss of cells that occurs between 24 hours and four weeks in cellular development (Olson *et al.*, 2006). While 12 days of enriched housing had no effect on proliferation in the DG, the number of surviving $BrdU^+$ cells after a further 29 days in these conditions was increased by 175% compared to control levels (Van Praag *et al.*, 1999b). Survival relative to proliferation was significantly higher than for the other groups explored, equating to 85%, as opposed to 56% in mice provided with a running wheel alone. Similarly, varying periods of EE (including a running wheel) did not influence proliferation in the DG when animals were killed one day after BrdU injections, but upregulated the survival of labelled cells after one month in these conditions (Kempermann *et al.*, 1997b; Auvergne *et al.*, 2002; Brown *et al.*, 2003a). The latter effect may be age-dependent, given that EE significantly enhanced survival of new neurons for six-month-old, but not 18-month-old, mice (Kempermann *et al.*, 1998). EE including a running wheel also enhances numbers of

immature differentiating neurons, with DCX^+ cell counts elevated after two months when compared to control-housed mice (Llorens-Martín *et al.*, 2007). Interestingly, individual differences in resultant AHN predicted depressive behaviour, as mice with more DCX^+ cells exhibited concurrently lower immobility during the FST.

An additional study measured the density of surviving $BrdU^+/NeuN^+$ neurons labelled six weeks previously, and found that this measure was increased in mice experiencing one week of EE that had ended between three and five, but not two, weeks previously (Tashiro *et al.*, 2007), when compared to constant control housing. This finding is consistent with the timescale for expression of NeuN in maturing neurons. Moreover, survival of new neurons that were produced during the four weeks of EE subsequent to injections was also higher for mice that were then moved to standard housing for an additional four months (Tashiro *et al.*, 2007), indicating stable neuronal integration that is not reliant on continued environmental complexity.

A few studies have explored the possibility of differential sensitivity of AHN across the septo-temporal hippocampal axis to exercise and EE, and there is evidence to support the particular influence of these experiences on the septal HF. Notably, four weeks of EE that included a running wheel led to upregulated numbers of both $Ki\text{-}67^+$ proliferating cells and DCX^+/$calretinin^+$ post-mitotic immature neurons, and promoted the four week survival of $BrdU^+$ cells, specifically within the septal mouse hippocampus (Tanti *et al.*, 2013). In a similar protocol, six weeks of EE was found to increase 24-hour BrdU-proliferation in both the septal and temporal DG, but to upregulate the one-month survival of $BrdU^+/NeuN^+$ neurons in the septal subregion alone (Tanti *et al.*, 2012). Furthermore, eight days of EE with running wheels increased DCX-immunoreactivity in the septal DG of female mice compared to the control group, but suppressed it in the temporal DG (Gualtieri *et al.*, 2017). It is possible that reduced neuronal differentiation in the temporal subregion related to stress associated with the protocol, or to the introduction of male urine to the bedding as part of enrichment. However, a final study instead found that the influence of one week of EE on later $BrdU^+/NeuN^+$ cell numbers was uniformly distributed along the septo-temporal axis (Tashiro *et al.*, 2007).

While most EE protocols have included access to a running wheel and thus the contribution of physical activity to resultant AHN levels, a few have specifically excluded a wheel from their EE apparatus. Bruel-Jungerman *et al.* (2005) placed groups of (otherwise pair-housed) rats in an enriched environment (with no running wheel) for just three hours a day over 14 days. After an intervening period of standard housing and cognitive testing, BrdU-labelled DG cells examined 9-13 days after injections were markedly increased compared to naïve controls. In

another study, EE without a wheel had a close to significant positive influence on the 35-day survival of pre-labelled $BrdU^+/NeuN^+$ cells, compared to standard housing (Fabel *et al.*, 2009). Net neurogenesis did not significantly differ from that in mice with mitotic cells labelled during 10 days of running, when this was followed by 35 days of standard housing, suggesting that these experiences have quantitatively similar effects. Moreover, the sequential combination of 10 days of running followed by 35 days of EE increased $BrdU^+/NeuN^+$ counts compared to the pairing of either condition with standard housing, indicating additive effects of exercise and enrichment (Fabel *et al.*, 2009). The extent to which EE can enhance net AHN thus appears to depend on the potential afforded by the pool of proliferative precursors.

Unlike AD treatment, running and EE appear to influence fate determination, and increase the proportion of new cells which differentiate into neurons (Kempermann *et al.*, 1998; Van Praag *et al.*, 1999b). For example, after two to four months of running, ~93% of $BrdU^+$ cells co-labelled for NeuN, compared to 82% for sedentary mice (van Praag *et al.*, 1999a). In these studies, astrocytic fate was not altered. Twenty-eight days of running also increased the percentage of $BrdU^+$ cells that co-localised with calretinin (Garrett *et al.*, 2012). However, 22 days of running did not alter the differentiation of the surviving cells into neurons (Castilla-Ortega *et al.*, 2014) and the proportion of calbindin co-expression after 68 days of EE with a running wheel was unaffected (Kempermann *et al.*, 1997b).

Certain studies have explored the comparative effects of exercise and EE on different subtypes of proliferating cell. While 24 hours of running increased the proliferation of type-2a and -2b $BrdU^+$ cells, with no influence on type-3, EE without a wheel only upregulated numbers of neuronal lineage-determined (type-2b and -3) progenitor cells (Steiner *et al.*, 2008). Following a longer period of four weeks, neither EE nor exercise affected the low proliferative activity of type-1 cells, while running again stimulated increased numbers of BrdU-labelled type 2 (a & b), but not type-3, cells (Kronenberg *et al.*, 2003). Consistent with other studies, EE was not found to increase the 24-hour proliferation of $BrdU^+$ cells, though both EE and running resulted in more newly-generated cells that expressed Prox-1, but not nestin, reflecting immature stages of granule cell development.

Concurrent with upregulated AHN, animals experiencing EE show relative improvements in spatial memory (Kempermann *et al.*, 1997b; Kempermann *et al.*, 1998; Bruel-Jungerman *et al.*, 2005). Moreover, the act of learning during hippocampal-dependent tasks is sufficient to promote the long-term survival of immature neurons in the GCL (but not the subventricular zone), without altering rates of proliferation (Gould *et al.*, 1999; Hairston *et al.*, 2005; Leuner

and Gould, 2010). Additionally, one exploration suggested that survival of new neurons was preferentially enhanced in the septal region of the rat GCL following spatial water-maze learning (Ambrogini *et al.*, 2000). Generally, it has been posited that stimuli with specific relevance to hippocampal functions, including spatial learning and navigating an enriched environment, preferentially modulate the recruitment of new neurons for long-term survival, with limited influence on proliferation (Fabel *et al.*, 2009). However, not all studies have produced such effects, with van Praag *et al.* (1999b) finding no influence of daily spatial water maze training on either cell proliferation, or survival one month later, in the mouse DG.

Generally, conditions that stimulate improvements in cognition for mammalian species tend to also have positive implications for mood (Sahay *et al.*, 2011). As reviewed, AD treatment, exercise and EE lead to improvements in behavioural measures of affective state in rats, mice and monkeys, and these experiences also upregulate AHN. Indirect evidence for the positive association between AHN and mood is therefore substantial. While less direct evidence is available, this is offered by ablation studies, which indicate that intact AHN is required for the positive behavioural effects of AD treatment, exercise and EE, particularly when ameliorating anxiety- and depressive-like symptoms induced by chronic stress (e.g. Santarelli *et al*., 2003; Li *et al*., 2008; Schloesser *et al*., 2010; Perera *et al*., 2011; Surget *et al*., 2011). Recent studies have further demonstrated that adult-born neurons play a role in suppressing the anxiogenic properties of mature granule cells, and this mechanism may at least partially explain the directional relationship between AHN and mood (Drew *et al*., 2016; Anacker *et al*., 2018). These findings are explored further in section 1.5.5, which summarises current understanding of the functions of newly-generated hippocampal neurons.

1.5.4 Mechanisms of regulation (& dysregulation) of AHN

Several proximal mechanisms by which experiential factors modulate AHN have been identified. Firstly, the glucocorticoid environment is a determining factor for the pre- and post-mitotic survival of neuronal progenitor cells in the adult hippocampus, as mediated through binding to MR and GR (Wong and Herbert, 2004; Wong and Herbert, 2005). While adrenalectomy increased the survival rate of post-mitotic progenitors at 28 days, administration of exogenous CORT during the post-mitotic period decreased survival (Wong and Herbert, 2004). Pharmacological antagonism of MR or GR had positive effects on proliferation, whereas agonists of MR and GR each reduced proliferation in adrenalectomised rats, and had additive suppressive effects when administered in combination (Wong and Herbert, 2005). GR expression has been detected in progenitor cells as early as four hours after BrdU-injection, and

around 50% of type-1, type-2a and type-3 cells were estimated to express the GR (Garcia *et al.*, 2004). Type-2b cells alone were generally devoid of GR expression, while concurrent MR expression was found only in mature calbindin-positive granule cells. This suggests that populations of developing neurons possess sensitivity to stress-induced, but not basal, concentrations of CORT. However, it is thought that regulation of AHN by CORT largely occurs indirectly, through binding to MR and GR in the surrounding local mature granule neurons and glial cells of the neurogenic niche (Wong and Herbert, 2005). Adult hippocampal astrocytes, for example, are known to be capable of regulating AHN, by instructing precursor stem cells to adopt a neuronal fate (Song *et al.*, 2002).

In addition to altering basal and stress induced levels of CORT, chronic stress may exacerbate dysregulation by altering the expression of MR and GR in the hippocampus. In human subjects, decreased hippocampal expression of *GR* (Webster *et al.*, 2002) and *MR* (López *et al.*, 1998; Klok *et al.*, 2011) mRNA has been observed in the post-mortem brains of patients with MDD. In rats, single prolonged stress downregulated both MR and GR hippocampal protein expression to different extents, resulting in a decrease in the MR/GR ratio (Zhe et al., 2008). Conversely, AD administration was found to elevate *MR* and *GR* mRNA expression in the rat hippocampus, but not in the parietal cortex (Seckl and Fink, 1992). Generally, the MR/GR balance is thought to be an important contributor to hippocampal dysfunction in the context of affective disorders (reviewed in Conrad, 2008), and may comprise an additional biomarker of cumulative stressful experience.

As discussed, manipulations associated with positive affect (e.g. exercise, social housing and sexual activity) may increase CORT, but still have positive effects on AHN (Stranahan *et al.*, 2006; Droste *et al.*, 2008; Kim *et al.*, 2013; Lehmann *et al.*, 2013). Stress-induced inhibition of AHN can also persist beyond the later normalization of CORT levels (e.g. Czéh *et al.*, 2002). Other mechanisms which modulate AHN levels may mediate the reviewed valence-specific relationship, which is dependent upon the context of the arousal-inducing experience. Stress and rewarding experiences differentially influence levels of various neurotransmitters (Sirgy, 2020), and there is evidence that GABA, serotonin, noradrenaline and dopamine are involved in the regulation of AHN (reviewed in Lucassen *et al.*, 2010). Similarly, stress reduces the expression of several growth and neurotrophic factors that positively influence neurogenesis, including brain-derived neurotrophic factor, insulin-like growth factor-1, nerve growth factor, epidermal growth factor, and vascular endothelial growth factor (reviewed in Schmidt and Duman, 2007). Conversely, positive experiences such as exercise stimulate the expression of these factors (Olson *et al.*, 2006; Wu *et al.*, 2007). Stress also leads to increases in glutamate

release through the activation of N-methyl-D-aspartate (NMDA) receptors, which have been identified on precursor cells (Gould *et al*., 1997; Lucassen *et al*., 2010). Astrocytes support the survival of developing neurons, and their numbers are also modulated by certain forms of experience (Lucassen *et al*., 2010). For example, fewer astroglia were detected in the adult tree shrew hippocampus following chronic psychosocial stress, and this effect was prevented by fluoxetine treatment (Czéh *et al*., 2006). EE also increased the number of astrocytes in the mouse DG (Fabel *et al*., 2009). Activation of microglia is additionally triggered by certain stressors, and initiates an inflammatory cascade with negative consequences for AHN (Tapp *et al*., 2019; Ekdahl *et al.*, 2003). Generally, the multiple complex factors that contribute to mediation of the relationship between experience and AHN may lend support to its status as an integrated proxy for animal welfare.

1.5.5 Potential functions of new hippocampal neurons

As reviewed, AHN responds dynamically to multiple extrinsic stimuli, with relevance to hippocampal functions associated with cognition, behaviour, affective pathophysiology and response to AD therapies (Encinas *et al.*, 2011). Experimental manipulations to suppress or upregulate AHN further support a direct causal contribution of adult-born cells to many of these functional domains.

In line with a crucial role in the formation of new episodic memories, the DG is thought to transform similar experiences into discrete, non-overlapping representations via a process known as pattern separation (Treves *et al.*, 2008). Transgenic ablation of AHN causes deficits in pattern separation (Tronel *et al.*, 2012), whereas efficiency in differentiating overlapping contextual representations is improved following genetic enhancement of the survival of adult-born neurons (Sahay *et al.*, 2011). Specifically, young neurons appear to activate inhibitory interneurons, which in turn inhibit the activity of mature granule cells, leading to the sparse DG activation required for efficient encoding of contextual representations (Anacker and Hen, 2017). Pharmacological suppression of AHN produces deficits in the acquisition of some, but not all, hippocampus-dependent learning tasks, with the involvement of new neurons apparently contingent upon the specific demands of each (Shors *et al.*, 2001; Shors *et al*., 2002; Deng *et al.*, 2010). Irradiation of AHN also indicates that neurons four to 28 days old at the time of training are required for long-term memory retention in the spatial water maze, but not to remember the location of a visibly cued platform (Snyder *et al.*, 2005). Contributions of young neurons to long-term memory formation may relate to the specific properties of long-term potentiation in these cells, which differ from those of mature neurons (Snyder *et al.*, 2005).

Ablation or inhibition of AHN concurrently precludes improvements in memory driven by environmental stimulation (Cao *et al*., 2004; Bruel-Jungerman *et al*., 2005; Hairston *et al*., 2005), while genetic upregulation of AHN improves spatial cognition (Cao *et al*., 2004). Additionally, new-born hippocampal cells are required for flexible spatial behaviour when the demands of a task are changed (or reversed). Ablation of AHN impairs learning of the goal location in a spatial water maze (Garthe *et al.*, 2009) or the rewarded corner of a cage (Kalm *et al.*, 2013) when this is altered from the initial criteria. Mice with ablated AHN also exhibit deficits in avoiding a rotating shock zone when this is added to a stationary zone learnt first, but not if this constitutes the first experience of place avoidance learning (Burghardt *et al.*, 2012). In contrast, EE stimulates AHN and also increases the efficiency of spatial search strategy employed to find a changed platform location in the water maze (Garthe *et al.*, 2009). It has been theorised that AHN may promote the erasure of previously learned associations, and thus facilitate the acquisition of novel associations by minimising proactive interference (Anacker and Hen, 2017).

The impairments in pattern separation and cognitive flexibility that occur after the ablation of AHN are also observed in human cases of depressive and anxiety disorders (Miller and Hen, 2015). It is posited that these deficits may lead to the generalisation of fearful and anxious associations across contexts after a stressor has ceased, as adult-born neurons are required both to erase these (flexibility) and to discretely encode new associations relating to safety (pattern separation) (Anacker and Hen, 2017). Several studies have explored a role of immature neurons in inhibiting anxiogenic responses. One study reported that transgenic mice with specific impairments in AHN exhibit a striking increase in anxiety-related behaviours in the EPM, light/dark box, and predator avoidance tests, compared to their wild-type counterparts (Revest *et al.*, 2009). However, most studies find that ablation of AHN does not increase depression or anxiety-like behaviors at baseline (e.g. Jayatissa *et al.*, 2009), but may potentiate these after stress (reviewed in Miller and Hen, 2015). Snyder *et al.* (2011) found no effect of ablating AHN on baseline behaviours, but observed an increase in depression- and anxiety-like behaviour following exposure to an acute moderate restraint stressor, as compared to control mice. AHN-deficient mice displayed increased NSF, increased immobility in the FST, and decreased sucrose preference in response to stress. Additionally, feedback inhibition to the HPA-axis was impaired, as CORT levels were slower to recover from stress and were less suppressed by GR agonist dexamethasone in AHN-ablated mice (Snyder *et al.*, 2011). Contribution of adult-born neurons to hippocampal regulation of the HPA-axis is further supported by the finding that

inhibition of AHN led to increased CORT release in response to the mild stress entailed by 15 minute exposure to a barren, brightly lit cage (Schloesser *et al.*, 2009).

Under non-stress conditions, experimentally increasing AHN also does not attenuate depressive immobility during the FST, or anxiety-like behaviour in an open field, light/dark box, EPM, or during NSF (Sahay *et al.*, 2011). However, in mice treated chronically with CORT, genetic upregulation of AHN is sufficient to reduce anxiety behaviour in an EPM and depressive immobility during the TST (Hill *et al.*, 2015). Moreover, optogenetic activation of adult-born neurons decreases activity of the DG under conditions of novelty and anxiety (via inhibitory interneurons), while activating a similar number of mature granule cells does not affect activity of the remaining mature cells (Drew *et al.*, 2016). It has subsequently been demonstrated that adult-born neurons (indirectly) inhibit the activity of mature granule cells in the temporal DG which are preferentially activated under anxiogenic conditions, and may thus contribute towards resilience to chronic stress (Anacker *et al.*, 2018).

In non-stress conditions, AD treatment reduces the latency of NSF behaviour, while irradiation of AHN prevents this effect (Santarelli *et al.*, 2003; Wang *et al.*, 2008). Genetic impairment of AHN that precluded stimulation by chronic AD treatment or voluntary running also rendered mice behaviourally insensitive to the reductions in NSF latency and TST immobility observed in wild-type mice after both treatments (Li *et al*., 2008). However, strain differences are apparent, as behavioural effects of ADs in unstressed BALB/cJ mice, which display a relatively high basal level of anxiety, do not require intact AHN (Holick *et al.*, 2007). Generally, activation of the stress axis may determine whether AD action relies on intact neurogenesis (Petrik *et al.*, 2012). Several studies indicate that, following exposure to chronic stress, mice with ablated AHN fail to exhibit reversal of anhedonia through monoaminergic AD treatment, supporting a causal role of the process in their therapeutic efficacy. After UCMS, ADs reduced latencies to groom when sucrose was placed on the snout, but irradiation prevented this effect (Santarelli *et al.*, 2003). Similarly, ablation of AHN prevented fluoxetine reversal of NSF (Surget *et al.*, 2008) and of inhibited consumption in the Cookie test of anhedonia (Surget *et al.*, 2011) for UCMS mice, and precluded AD protection from depressive behavioural symptoms in bonnet monkeys exposed to separation stress (Perera *et al*., 2011). Fluoxetine also reduces FST immobility in a manner blocked by irradiation (Airan *et al.*, 2007). Furthermore, the ability of fluoxetine to restore hippocampal regulation of the HPA-axis under conditions of chronic stress is contingent upon the presence of an intact neurogenic niche (Surget *et al.*, 2011). Interestingly, the time course for AD drugs to orchestrate significant improvements in rodent behavioural symptoms following stress corresponds with the time taken for newly generated

neurons to become functionally integrated (Aniol and Gulyaeva, 2015). However, certain non-monoaminergic compounds produce AD-like behavioural effects even in irradiated stressed mice (Nollet *et al.*, 2012).

Lastly, the recovery of behavioural submissive symptoms resulting from psychosocial stress that occurs in the majority of mice transferred to an enriched environment is prevented by selective ablation of AHN (Schloesser *et al.*, 2010). While EE increases the survival of immature neurons in the temporal DG following chronic social defeat, this effect is prevented by adrenalectomy (Lehmann *et al.*, 2013). Concurrent with suppressing AHN, this procedure severely disrupted the recovery of behavioural symptoms, whereas defeated mice with intact adrenals, and thus neurogenic responses to enrichment, displayed post-EE behavioural responses comparable to non-defeated controls. AHN thus appears to play a causal role in the non-pharmacological restoration of normal behaviour following an induced negative mood state. Overall, experimental manipulations to ablate or upregulate AHN support multiple contributions of adult-born neurons to mechanisms of affective regulation and cognitive processing, pointing to the importance of the associated plasticity for hippocampal-dependent functions.

1.5.6 Does AHN integrate sequentially occurring experiences?

Overall, a wealth of evidence supports the responsivity of AHN to various modalities of welfare-relevant experience, with the direction of this modulation depending upon their valence. Ideally, a marker of long-term welfare would fulfil the additional criterion of quantitatively integrating positive and negative experiences that occur simultaneously or sequentially during an animal's lifetime.

Simultaneous stress and exercise appear to result in levels of proliferation that are intermediate to either experience alone, and comparable to control animals. For example, exercise restored the reduction in $BrdU^+$ proliferation (measured one week post-injection) that resulted from four weeks of CUS alone to control levels (Kiuchi *et al.*, 2012). A moderate daily dose of exogenous CORT (40 mg/kg) also reduced two to five day $BrdU^+$ proliferation, but combined voluntary running over 28 days resulted in a number of labelled cells intermediate to either running or CORT alone (Yau *et al.*, 2012). An eight week regime of restraint stress and wheel access similarly resulted in levels of Ki-67 staining equivalent to control mice, whereas these were suppressed by restraint alone (Nakajima *et al.*, 2010). However, the three-week survival of $BrdU^+$ cells was still decreased in restraint with exercise. A further study found that stress resulting from individual housing delayed the positive effects of running on hippocampal

proliferation, which were not observable until after 48 days of exercise, but were detectable after 11 days in group-housed rats (Stranahan *et al.*, 2006). In a study of learning, mice trained on the hippocampal-dependent hidden platform water maze exhibited levels of surviving pre-labelled $BrdU^+$ new neurons and type-3 precursor cells that were intermediate to higher levels in untrained animals and lower levels in mice trained on a visually cued, hippocampal-independent variant (Ehninger and Kempermann, 2005). This finding was attributed to a combination of stress and learning/exercise entailed by the training, as providing three days of pre-exposure to the pool precluded these group differences. Finally, simultaneous opportunities for sexual activity, a primary reinforcer associated with a positive emotional response, counteracted the suppression in survival of pre-labelled $BrdU^+$ cells caused by 21 days of restraint stress (Kim *et al.*, 2013), with resultant levels comparable to control animals and intermediate to those in mice experiencing sexual activity alone.

In line with its causal role in facilitating the recovery of anxious and depressive behaviour from chronic stress (Santarelli *et al.*, 2003; Airan *et al.*, 2007; Surget *et al.*, 2008; Schloesser *et al.*, 2010; Surget *et al.*, 2011; Lehmann *et al.*, 2013), successive experiences appear to have additive or compensatory effects on levels of AHN, depending on whether their valence is shared or opposing. Rats exposed to 10 days of EE had higher two-hour $BrdU^+$ proliferation in the SGZ/GCL, while rats subjected to 21 days of restraint stress followed by 10 days of EE housing had subsequent levels equivalent to unstressed, standard-housed controls (Veena *et al.*, 2008). Restraint stress also decreased the 31-day survival and proportional differentiation of BrdU-labelled cells, an effect which was ameliorated by 10 days of subsequent EE to produce levels comparable to control animals (Veena *et al.*, 2009). Voluntary exercise beginning 7 days before chronic restraint and continued in parallel for 18 days resulted in survival of pre-labelled $DCX^+/BrdU^+$ double-labelled cells intermediate to exercise or stress alone and similar to controls, though $BrdU^+/Calbindin^+$ neurons were higher in the combined stress/exercise than for the other groups (Castilla-Ortega *et al.*, 2014). Likely due to independent effects on the proliferation and survival of new neurons, whereas housing in an enriched cage containing a running wheel does not elevate AHN more than running alone (Van Praag *et al.*, 1999b), a period of 10 days of running exercise followed by 35 days of EE has an additive effect - enhancing cell survival significantly more than either experience alone (Fabel *et al.*, 2009).

Collectively, these studies suggest that levels of AHN may reflect the integrated compensatory or additive effects of experiences associated with longer-term moods, on a relatively cumulative basis. Survival of new neurons is typically measured four-weeks after BrdU injection, by which time the majority of persisting cells co-express mature neuronal marker NeuN (Cameron *et al.*,

1993) and are likely to still be present 11 months later (Kempermann *et al*., 2003). Experience within the last month of life is therefore likely to be reflected by the number of surviving immature or young neuronal cells present at the time of an animal's death. Measures of proliferation may reflect more recent experience, as the majority of cells reach the post-mitotic stage within three to seven days of the initial division (Kempermann *et al*., 2004; Encinas *et al*., 2011). Certain experiences have been shown to influence ongoing rates of proliferation (Malberg and Duman, 2003; Elizalde *et al*., 2010; Perera *et al*., 2007) and survival (Elizalde *et al*., 2010) a month later, without continuation during this time, while in other cases, proliferation returns to baseline quickly (i.e. within 24-hours) (Lagace *et al*., 2010). Aside from the influence of exercise (Kronenberg *et al*., 2006; Snyder *et al*., 2009), evidence in rats and mice does not suggest that AHN habituates to sustained experiences, and levels may remain suppressed at times when CORT levels have normalized following stress (Czéh *et al*., 2002; Lucassen *et al*., 2010). Studies in rodents do not appear to have yet explored whether continuously prolonged stress leads to increasing declines in immature neuronal cells with time, though cell numbers have been found to linearly predict levels of depressive-like behaviours (Jayatissa *et al*., 2006; Llorens-Martín *et al*., 2007). Overall, while post-mortem AHN would not be expected to reflect the experience of past stresses that ceased to occur more than one to two months ago, cell numbers should reflect recurrent experiences that continued into the previous one to two months of life. In the case of adult laying hens, many notable welfare issues (e.g. relating to housing, keel bone fractures and feather-pecking) are likely to be chronic in nature. Unlike plasma CORT (Mench *et al*., 1986), measurements of neuronal differentiation and survival are not likely to be confounded by acute stress associated with sampling.

1.6 The Avian Hippocampal Formation

1.6.1 Neurogenesis in the avian brain

In the mammalian telencephalon, the lateral ventricle separates an inner region of nuclear grey matter (or basal ganglia) ventrally from an outer region of layered grey matter dorsally. In non-mammals, both the inner and outer parts of the telencephalon are mainly composed of nuclear grey matter, lying mostly beneath the lateral ventricle in reptiles and birds (Jarvis *et al.*, 2005). As such, the avian cerebrum was once thought to consist almost entirely of basal ganglia, termed palaeoencephalon (old brain) and associated with the coordination of instinctive behaviour. However, it is now widely recognised that, despite its nuclear organisation, the avian cerebrum has a large pallial, or cortical-equivalent, territory that supports cognitive functions similar to those of the laminar mammalian cortex (Jarvis *et al.*, 2005).

In adult mammals and birds, neurogenesis occurs primarily within regions of the telencephalon (Alvarez-Buylla *et al.*, 1994), but also in discrete areas of the diencephalon (e.g. the hypothalamus (Recabal *et al.*, 2017)) and metencephalon (e.g. the cerebellum (Ponti *et al.*, 2008; Mezey *et al.*, 2011)). In the avian telencephalon, radial cells are found alongside non-dividing ependymal cells in the walls of the lateral ventricle, or the ventricular zone (VZ), and continue to undergo mitosis after development (Alvarez-Buylla *et al.*, 1990). These primary neural precursors are frequently characterised by long basal processes and are organised as a columnar epithelium, wherein every cell contacts the ventricle (Alvarez-Buylla *et al.*, 1998). VZ radial cells are rich in vimentin, an intermediate filament protein expressed by all neuroepithelial cells, but despite having a morphology similar to rodent type-1 radial glia, do not express GFAP (Alvarez-Buylla *et al.*, 1990). All cells labelled one to two hours after a single injection of thymidine-^{3}H are of this type (Alvarez-Buylla *et al.*, 1998). Within the VZ are highly proliferative regions of clustered radial cells, known as hot spots (Alvarez-Buylla *et al.*, 1990), which are concentrated at the ventral and dorsal reaches of the lateral wall of the lateral ventricle (Nottebohm and Alvarez-Buylla, 1993). Three days after administration of thymidine-^{3}H, small labelled fusiform cells are first observable adjacent to the lateral wall of the lateral ventricle, in greater numbers close to the hot spots (Alvarez-Buylla *et al.*, 1990; Nottebohm and Alvarez-Buylla, 1993). These cells are never found in contact with the ventricle and exhibit microtubule-rich tangential processes, consistent with on-going migration. Following survival times of five, 24, and 74 hours after thymidine-^{3}H injection, the proportion of labelled radial cells is increasingly reduced in favour of these fusiform cells, which accounted for ~73% by the latter time-point (Alvarez-Buylla *et al.*, 1998). On successive days, these daughter cells are found at ever-increasing distances from the ventricular wall, with their migratory pathways often guided by the processes of the radial cells (Nottebohm and Alvarez-Buylla, 1993). Thymidine-^{3}H-labelled mature neurons are first found 20 days after injection in the adult canary telencephalon, suggesting this is the minimum time required for neuronal differentiation in this species (Nottebohm and Alvarez-Buylla, 1993). The total number of migrating neural cells also peaks at day 20, while one-third of these cells remain detectable at 40 days post-injection (Alvarez-Buylla and Nottebohm, 1988). Similar to mammalian AHN, a large number of immature neurons thus appear to die before terminal differentiation (Kempermann *et al.*, 2004).

Compared to the mammalian brain, the migratory pathways of differentiating immature neurons are diffuse (Alvarez-Buylla and Nottebohm, 1988; Melleu *et al.*, 2013), and their incorporation into functional circuits occurs in more numerous brain areas (Melleu *et al.*, 2016). As in

mammals, DCX comprises an endogenous marker of developing immature neurons in the avian brain, with conserved functions relating to migration and differentiation (Hannan *et al.*, 1999; Balthazart *et al.*, 2008; Mezey *et al.*, 2011). However, one study reported DCX expression in active adult neurons of up to a year old in the adult canary brain (Vellema *et al*., 2014), leading the authors to dispute the specificity of the marker to immature neurons. Widespread DCX-expression can be detected throughout all forebrain areas and in the cerebellum of 14-week-old domestic chicks, but not within any midbrain or brainstem regions (Mezey *et al.*, 2011). Similar patterns of staining are observed in adult pigeons (Melleu *et al.*, 2013) and canaries (Boseret *et al.*, 2007), with the nidopallium and enclosed high vocal centre displaying a particularly high neurogenic density in the songbird. Whereas transient DCX expression lasts for an average of around two weeks in adult rodents (Brown *et al.*, 2003a), DCX expression in passerine birds persists for 25 to 30 days after a new neuron is born (Balthazart *et al.*, 2008). DCX^+ cells with a fusiform (or bipolar) morphology, associated with migration, co-label more with BrdU at 10 days post-injection (Balthazart *et al.*, 2008), while multipolar DCX-immunoreactive cells are also widely observed (Melleu *et al.*, 2013) and co-label with BrdU more frequently at day 30 (Balthazart *et al.*, 2008). The latter cells are thus presumed to be more mature and settling (Boseret *et al.*, 2007). In multiple avian species, the HF has been reported to comprise a proliferative hot spot, incorporating BrdU and endowed with DCX^+ cells of both morphologies (Patel *et al.*, 1997b; LaDage *et al.*, 2010a; LaDage *et al.*, 2010b; Law *et al.*, 2010; Sherry and Hoshooley, 2010; Melleu *et al.*, 2013).

1.6.2 Location & structure of the avian hippocampal formation

Similarities in development, connectivity, neuromorphology, and neurochemistry support homology of the avian HF to the mammalian hippocampus (Colombo and Broadbent, 2000; Atoji and Wild, 2006; Abellan *et al.*, 2014; Mayer *et al.*, 2015). Like the mammalian structure, the avian HF occupies a dorsomedial position in the telencephalon (Striedter, 2016). Specifically, the HF is located at the dorsomedial wall of the external telencephalic surface, superior to the lateral ventricle. Indeed, the VZ extends along the entire rostro-caudal extent of the HF (Patel *et al.*, 1997b). Following 300 million years of independent evolution (Striedter, 2016), internal cytoarchitecture of the avian HF differs markedly from the mammalian hippocampus. Most obviously, the avian HF lacks distinctly identifiable morphological subdivisions including the DG and Ammon's horn (Colombo and Broadbent, 2000) and comprises a nearly homogeneous arrangement of neurons (Gupta *et al.*, 2012). It has been suggested that extensive intrahippocampal collateral axon branches displayed by many neurons in the avian HF (Tömböl *et al.*, 2000) resemble the extensive collaterals in area CA3 of the

mammalian hippocampus (Striedter, 2016). However, Golgi and Timm's staining reveals an absence of mossy fibres (Striedter, 2016), while granule cells (unique in the mammalian brain for their expression of Prox-1 during adulthood (Karalay *et al*., 2011)) are not present. Nevertheless, embryonic expression of Prox-1 in the tip of the avian HF supports homology with the DG (Abellan *et al*., 2014).

Proposed subdivisions of the avian HF have largely stemmed from characterisation of the pigeon brain and differ between authors. However, three major hippocampal divisions, likely common to all avian species (Striedter, 2016), are the ventral, dorsomedial, and dorsolateral regions (Székely and Krebs, 1996; Kahn *et al.*, 2003; Atoji and Wild, 2004). In contrast, smaller subdivisions within these areas may be species-specific. Notably, the V-shaped layer identifiable within the ventral hippocampus of pigeons and songbirds is indistinct or absent in many other bird species, including chickens, ostriches, parrots, and suboscines (Striedter, 2016). Generally, the same internal structure is consistently maintained across the longitudinal axis of the HF, though all divisions are angled from ventromedial in rostral sections to dorsolateral in more caudal sections (Erichsen *et al.*, 1991; Smulders, 2017). Homologies between avian and mammalian HF subfields remain an area of much debate. For example, while authors present various arguments for homology of the dorsomedial (Székely and Krebs, 1996; Kahn *et al.*, 2003), ventral (Gupta *et al.*, 2012), or internal V-shaped (Atoji *et al.*, 2016) regions of the avian HF to the mammalian DG, there are a lack of uniquely shared features, and a homologue does not necessarily exist (Striedter, 2016).

Pathway tracing studies in pigeons have indicated that the overall efferent and afferent connectivity of the avian HF is remarkably similar to that of its mammalian counterpart (Jarvis *et al.*, 2005; Atoji and Wild, 2006). Internally, the ventral, dorsomedial, and dorsolateral divisions are reciprocally connected (Striedter, 2016). Extrinsic inputs originate mainly in the hyperpallium (Reiner *et al.*, 2004), an area homologous to part of the neocortex, which conveys highly processed, multimodal sensory information to the HF. Further inputs arise from regions of the nidopallium that process somatosensory and auditory information, and from the dorsolateral corticoid area, which receives inputs from a multitude of brain regions, including the primary olfactory cortex (Atoji and Wild, 2005; Striedter, 2016). Inputs from beyond the telencephalon derive mainly from the dorsal thalamus (Atoji and Wild, 2004) and the supramammillary region of the hypothalamus (Berk and Hawkin, 1985). Both the avian and mammalian HF receive projections from the contralateral hippocampus, diagonal band, locus coeruleus, and raphe nucleus (Colombo and Broadbent, 2000). Efferent projections extend predominantly from the dorsomedial and dorsolateral HF and tend to reciprocate the major

afferents (Striedter, 2016). The dorsomedial division also projects heavily to the septum, and has direct projections to the lateral hypothalamus (Székely and Krebs, 1996; Atoji and Wild, 2004).

1.6.3 Evidence for functional similarity to the mammalian hippocampus

Like the mammalian hippocampus, the avian HF appears to play a key role in spatial-cognitive processing. Following the observation that large bilateral lesions of the HF impair the ability of pigeons to return to their home loft (Bingman *et al.,* 1984), several studies in homing pigeons have supported the involvement of the avian HF in spatial navigation. Specifically, while navigational map and compass mechanisms used to home from more distant, unfamiliar areas are not generally affected by HF lesions, damaged birds are impaired during the portion of the flight expected to be guided by the location of familiar visual landmarks (reviewed in Bingman *et al.*, 2005). Lesioned pigeons are also less successful than control birds in re-orienting homewards following an initial navigational error, suggesting that the HF supports a map-like representation of familiar landmarks and landscape features, which may span tens of kilometers (Gagliardo *et al.*, 2009).

In domestic chicks, the HF is involved in the representation of environmental shape and processing of spatial-geometric information. Neural activation in the HF, reflected by c-Fos expression, was greater following exposure to enclosures of two different shapes (square and rectangle), than to either shape twice (Mayer *et al.*, 2018). HF c-Fos expression was also higher in chicks trained to locate the rewarded corners of a rectangular arena according to its shape when compared to chicks trained to discriminate local features in a square-shaped arena (Mayer *et al.*, 2015). The latter task preferentially activated a different area, within the medial striatum.

The HF is also necessary for food-storing birds to remember cache locations. Though rates of caching and frequency of retrieval attempts were not influenced by bilateral HF lesions, this damage severely impaired the ability of chickadees to retrieve seeds that they had cached in an indoor aviary, reducing accuracy to chance levels (Sherry and Vaccarino, 1989). Eurasian nutcrackers (*Nucifraga caryocatactes*) with HF lesions were able to remember cache locations for about 15 minutes, but could not retrieve caches they had stored 1–3 hours earlier (Krushinskaya, 1970). In contrast, lesions to the visual Wulst in the hyperpallium did not affect cache retrieval in either study. In intact black-capped chickadees (*Poecile atricapillus*), HF immediate early gene expression correlates with memory and accuracy of retrieval during a food-hoarding task (Smulders and DeVoogd, 2000), suggesting that additional neurons are recruited to the active network during spatial memory processing.

Even in non-hoarding species, the avian HF is crucially involved in learning and memory for spatial information. Similar to findings in mammals, lesions to the pigeon HF specifically impair place learning in an open field (Colombo *et al.*, 1997) or a dry version of the Morris water-maze (Fremouw *et al.*, 1997), but do not inhibit visual discrimination or learning based upon a cue in these respective tasks. In chickadees, HF lesions also inhibit learning of a goal location on a computer screen, but do not influence memory for a colour cue (Hampton and Shettleworth, 1996). Damage to the HF further produces deficits in the performance of a spatial variant of the delayed match-to-sample task when retention intervals are long (Good and Macphail, 1994). Ibotenic acid has also been utilised to make excitotoxic lesions that spare the fibres of axons passing through the HF. This manipulation still results in spatial memory deficits for Zebra finches (*Taeniopygia guttata*) (Patel *et al.*, 1997a; Bailey *et al.*, 2009) and impaired acquisition of repeated spatial discriminations in pigeons (Watanabe, 2006), indicating causal involvement of neurons of the HF itself. Moreover, HF-lesioned zebra finches given transplants of embryonic hippocampal tissue, but not anterior telencephalon tissue, showed a significant reversal of the performance deficits on the spatial memory task (Patel *et al.*, 1997a).

Temporary bilateral activation of the chickadee HF with lidocaine impairs the acquisition of spatial memories, but only when the locations could not instead be identified by local colour cues (Shiflett *et al.*, 2003). Furthermore, inactivation of the HF during memory retrieval impaired the ability of birds to find locations they had learned around 15 minutes earlier, but they did not impair memory retrieval 3 hours after learning. Secondly, infusion of an NMDA receptor antagonist into the chickadee HF blocked the formation of long-term, but not short-term, spatial memories, tested 3 hours versus 15 minutes after learning respectively (Shiflett *et al.*, 2004). Together, these findings indicate that, like rodents (Snyder *et al.*, 2005), songbirds require the HF to encode long-term spatial memories, but recall of these memories can become independent of the hippocampus after several hours (Striedter, 2016). Overall, both birds and mammals with damage to the hippocampus are severely impaired in a variety of spatial tasks, such as navigation, maze learning, and the retention of spatial information, but perform a variety of visual tasks normally (Colombo and Broadbent, 2000).

Lesions to the HF also influence certain non-spatial behaviours. A phenomenon known as autoshaping occurs when pigeons are presented with a non-reinforced stimulus that, after a few seconds, is followed by the delivery of food. After a number of these pairings, pigeons will reliably peck at the stimulus, in a response strong enough to persist even when pecks are actively punished by not providing food (Williams and Williams, 1969). Pigeons with lesions to the HF are slower to acquire an autoshaped response and emit subsequently fewer pecks to the stimulus

than intact birds (reviewed in Colombo and Broadbent, 2000), in an effect comparable to that observed in hippocampus-lesioned rats (Reilly and Good, 1989). As autoshaping is context-dependent (Honey *et al.*, 1990), this deficit may relate to the role of the HF in processing contextual information. The HF has also been argued to form a key component of a behavioural inhibition system, as rats with hippocampal lesions continue to respond in situations typically associated with suppression, such as during passive avoidance, extinction of conditioned responses, contextual freezing and distraction by external or novel stimuli (reviewed in Colombo and Broadbent, 2000). Pigeons with HF lesions are also less distracted by the presence of an experimenter during T-maze training (Colombo and Broadbent, 1999), and do not adjust the number of pecks distributed towards target stimuli based upon their differing probabilities of reinforcement (Scarf *et al.*, 2014). The HF is likewise involved in passive avoidance learning in chickens (Unal *et al.*, 2002; Nikolakopoulou *et al.*, 2006; Krause *et al.*, 2008) and classical aversive conditioning (tone-shock association) in pigeons (Brito *et al.*, 2006).

Whilst the avian HF has direct projections to the hypothalamus (Székely and Krebs, 1996; Atoji and Wild, 2004), it is not directly connected to the PVN, which regulates the HPA stress-axis (Smulders, 2017). Similar to the mammalian brain, indirect connections may exist via a number of pathways (reviewed in Smulders, 2017), including through other hypothalamic nuclei, such as the lateral hypothalamus (Székely and Krebs, 1996; Atoji and Wild, 2004), via the medial and lateral septal nuclei (Atoji and Wild, 2004), the nucleus of the diagonal band (Krayniak and Siegel, 1978) and the BNST (Atoji and Wild, 2006). Experiments in pigeons indicate that hypothalamic control of CORT release is homologously mediated by the avian HF. HF lesions result in significantly increased baseline levels of circulating CORT, which are continuously maintained at levels comparable to the peak of the normal circadian cycle (Bouillé and Baylé, 1973). This chronic elevation is accompanied by a lower CORT response to restraint stress, presumed to be a consequence of stronger negative feedback. Moreover, 10 minutes of electrical stimulation of hippocampal neurons acted to suppress baseline plasma CORT concentrations. Recordings of multi-unit activity confirmed the role of the HF in regulating the circadian activity of the hypothalamus, which increases when HF firing rates drop two to three hours prior to the morning CORT peak (Bouillé and Baylé, 1976). These studies demonstrate that the HF inhibits activity of the HPA-axis in birds, as it does in mammals (Smulders, 2017). In addition to the plentiful evidence for the role of the avian HF in spatial-cognitive processing and memory, there is thus also support for a contribution to aspects of affective regulation mirroring the mammalian hippocampus.

1.6.4 Evidence for sensitivity of AHN in the avian HF to experience

Like the mammalian hippocampus, the avian HF is one of few brain regions to strongly express high-affinity MR receptors, along with the more widely distributed GR receptors (Dickens *et al.*, 2009). As in mammals, the MR/GR expression ratio in the HF of birds is sensitive to stress, and is altered by UCMS in European starlings (*Sturnus vulgaris*) (Dickens *et al*., 2009) and maternal deprivation in zebra finches (*Taeniopygia guttata*) (Banerjee *et al*, 2011). Given that avian hippocampal neurons are similarly sensitive to CORT, it is possible that mechanisms including stress-induced CORT release may lead to downregulation of levels of AHN in birds, as occurs in rodents (Lehmann *et al.*, 2013). Though fewer studies have explored this possibility than for mammalian species, there is accumulating evidence to suggest that neurogenesis in the adult avian HF is also suppressed by chronic stress.

Firstly, single trial exposure to a bitter tasting bead resulted in a decrease in the number of BrdU-labelled proliferating cells present in the HF of domestic chicks 24 hours later, compared to their water-trained conspecifics (Nikolakopoulou et al, 2006). The interpretation that the bitter substance evoked an acute stress response was supported by an observed elevation in CORT levels following this avoidance learning. The effect was limited to proliferation, as numbers of surviving $BrdU^+$ cells measured nine days after training did not differ between the two groups. Secondly, low social status may be associated with chronic stress, and subordinate captive mountain chickadees (*Poecile gambeli*) exhibit significantly lower proliferation in the hippocampal VZ two days after BrdU injection than their dominant enclosure-mates (Pravosudov and Omanska, 2005). Neither HF volume, nor the total number of neurons, differed between dominant and subordinate birds, while relative survival of immature neurons was not compared. However, social isolation has been associated with reduced long-term survival of young neurons in the zebra finch HF. Following administration of thymidine-^{3}H at four to five months of age, birds transferred to be housed in aviaries alone exhibited fewer surviving new neurons after 40, 60 and 150 days than birds transferred to equivalent enclosures with a group of conspecifics (Barnea *et al.*, 2006).

A group of studies has explored the influence of captivity, which likely constitutes a source of chronic stress, on levels of AHN in wild-caught chickadees. Barnea and Nottebohm (1994) noted that captive adult black-capped chickadees had fewer surviving new neurons incorporated into the HF six weeks after thymidine-^{3}H administration than conspecifics living in the wild. Strikingly, levels of neuronal addition in the captive birds were only half as high. Similarly, wild-caught mountain chickadees kept in captivity for around four months were found to have a significantly lower number of immature DCX^+ neurons in the HF than free-ranging

conspecifics sampled directly from the wild (LaDage *et al.*, 2010a), with proportions of differentiating neurons ranging from 9% to 31% respectively. However, levels of DCX^+ neurons in the HF of black-capped chickadees reared by hand were similar to those in their free-living counterparts, despite the former group having a markedly smaller hippocampal volume (Roth *et al.*, 2012). As the dramatic environmental changes entailed by captivity are presumably stressful for wild birds, whereas hand-reared individuals would be accustomed to these conditions (Barnea and Pravosudov, 2011), these observations point collectively to the sensitivity of AHN in the avian HF to chronic stress. The observed differences in hippocampal volume, on the other hand, may relate to the experience of environmental complexity or stimulation, given that the laboratory setting is relatively impoverished. While hand-reared birds did not have a lower estimated total number of HF neurons than their free-living conspecifics (Roth *et al.*, 2012), other experience-dependent structural modifications may occur during development. For example, increasing the complexity of the environment of domestic chicks through inclusion of a visual barrier led to development of hippocampal pyramidal cells with longer dendrites with more spines (Freire and Cheng, 2004). Conversely, another study found no detrimental impact of captivity on the four to six week survival of BrdU-labelled newborn cells in black-capped chickadees (Tarr *et al.*, 2009). It is possible that this discrepancy relates to the smaller population of neurons identified using a single injection of BrdU than by DCX staining, whilst sensitivity of the technique employed to detect labelled cells may differ from that afforded by silver grain autoradiography (see Smulders, 2017).

A few studies have exposed avian species to experimental chronic stress and measured the consequences for AHN. In rodent models of mood disorders, altered or unpredictable light cycles are often utilised as a stressor. Following housing in an environment of constant light for two weeks, diurnal Indian house crows (*Corvus splendens*) exhibited significantly fewer DCX-immunoreactive cells in the HF when compared to control crows kept on a 12:12 light-dark cycle (Taufique *et al.*, 2018). This reduction in neuronal differentiation was accompanied by impaired cognitive performance, indicated by more errors in tasks of spatial and pattern association. The constant light conditions were associated with a significant reduction in rest periods, while sleep-deprivation in rats is known to markedly suppress cell proliferation in the DG (Mueller *et al.*, 2008). For commercial broiler breeder hens, the restricted diet required to protect physical health contrasts with a strong motivation to eat, resulting in chronic hunger that likely causes stress. Compared to chickens released from restriction with *ad libitum* feed from seven to 12 weeks of life, birds feed-restricted for 12 weeks displayed elevated CORT and a significantly lower density of BrdU-labelled cells surviving a week post-injection, while

hippocampal volume and the total number of neurons were unaffected (Robertson *et al.*, 2017). As indices of brain size were not impacted by food-restriction, this effect does not appear to be a result of general nutritional restriction of neural development. In order to mirror protocols employed in rodent models, one study exposed adult laying hens to an eight-week program of UCMS. Compared to control hens, birds exposed to this sequence of stressors exhibited a selective decrease in the number of DCX^+ differentiating neurons in the caudal HF (Gualtieri *et al.*, 2019). This preferential sensitivity may support homology of the caudal chicken HF to the temporal subregion of the rodent hippocampus (discussed further in section 1.6.5).

Just as EE stimulates AHN in the rodent brain, generalised environmental complexity is observed to augment the number of new hippocampal neurons generated in adult birds (Barnea and Nottebohm, 1994). Notably, the passerine HF undergoes changes in both size and rates of AHN that correlate with seasonal patterns in food-storing behaviour (see Sherry and Hoshooley (2010) for a review). For example, black-capped chickadees have more six-week surviving thymidine-^{3}H-labelled neurons in October, when hoarding peaks, than in August or February-March (Barnea and Nottebohm, 1994). This use of hippocampal-dependent memory has been demonstrated to causally modulate AHN in laboratory settings, where hoarding opportunities can be manipulated. Immediately following their third session of caching and retrieving food, hand-reared marsh tits (*Parus palustris*) exhibited greater two-hour proliferation of thymidine-^{3}H-labelled cells in the hippocampal VZ than control birds given powdered food that could not be cached (Patel *et al.*, 1997b). A relative increase in the total number of HF neurons was also observed 15 days later in birds provided with eight total trials of caching experience (once every three days). In a similar manipulation, mountain chickadees afforded caching experience on alternate days for several months had subsequently more immature neurons expressing DCX, and a greater proportion of DCX^+ neurons, than deprived birds (LaDage *et al.*, 2010a). The number and density of DCX^+ neurons in the hyperpallium apicale, an area laterally adjacent to the HF, did not differ between groups. The authors confirmed in an additional subset of birds that distances travelled within the testing room were not lower for birds restricted from caching, indicating that differential physical activity was not responsible for this effect. Whether physical exercise indeed stimulates AHN in birds is currently unclear. Flight tunnel exercise was found to have no influence on DCX^+ cell numbers in European starlings (*Sturnus vulgaris*) (Hall *et al.*, 2014), though it is possible that the large home aviaries common to control birds afforded plentiful opportunity for voluntary exercise in both groups. Enriched housing, however, has been demonstrated to upregulate AHN in hand-reared pigeons. Following six weeks of housing in slightly larger cages that contained toys and salient visual stimuli and

provided visual contact with neighbours, a greater number of DCX-immunoreactive cells were observed in the HF than for control pigeons kept in standard cages (Melleu *et al.*, 2016).

There is thus convincing evidence that AHN in the avian brain is stimulated by cognitive enrichment and spatial memory demands under conditions of environmental complexity. However, whether these conditions have positive implications for mood, as is the case in mammalian species, is less clear. This notion is supported by the observation that providing EE for domestic chicks reduced general fearfulness reflected by a broad range of measures, including behaviour in an open field and decreased avoidance of a novel object or experimenter (Jones and Waddington, 1992). However, immobility behaviour, thought to reflect anxiety, was actually enhanced in pigeons with AHN stimulated by EE (Melleu *et al.*, 2016). It is possible that this response may have related to a greater contrast between housing and test conditions than for standard-housed birds. Further evidence for conservation of the relationship between stimulated AHN and positive affective experience in avian species may be required.

1.6.5 Does a functional gradient exist across the rostro-caudal axis of the avian HF?

In the mammalian hippocampus, functional specialisation exists across the longitudinal axis, perpendicular to major subdivisions such as the DG and Ammon's horn (see section1.3.2). The septal subregion physically connects to the septum and is involved predominantly in spatial-cognitive functions, while the temporal subregion has a greater role in affective modulation. As the rostral end of the avian HF similarly connects to the septum (Atoji and Wild, 2006) and the same general subdivisions (dorsolateral, dorsomedial and ventral) are present in all coronal sections moving caudally (though the entire structure shifts somewhat from medial to lateral), it is possible that an equivalent axis exists in the avian brain. While this possibility has been explored by few studies, patterns in connectivity, gene expression, AHN and modulation of the HPA-axis provide some support (reviewed in Smulders, 2017; Herold *et al.*, 2019).

In general, the majority of external connections to the HF do not differ along its rostro-caudal axis, though projections to the contralateral HF are topographically arranged, connecting rostral to rostral and caudal to caudal (Atoji *et al.*, 2002; Atoji and Wild, 2004). Projections from the HF to the septal nuclei are also topographically organized (Herold *et al.*, 2019), with more rostral areas projecting to the nucleus of the diagonal band and the rostral septum, whilst the caudal HF projects to the post-commissural septum (Atoji *et al.*, 2002; Atoji and Wild, 2004; Smulders, 2017). Input from nucleus Taeniae of the amygdala largely enters the middle third of the HF, and is absent from the rostral third (Atoji *et al.*, 2002). Axon paths also differ, as the caudal HF sends axons dorso-laterally around the lateral ventricle, while rostral axons run

ventro-medially (Atoji *et al.*, 2002). Unlike in the mammalian hippocampus, projections to the BNST arise from across the rostro-caudal axis, rather than originating preferentially from the temporal pole (Atoji and Wild, 2006; Smulders, 2017). Most relevant activation or inactivation studies have non-selectively targeted the entire rostro-caudal extent of the avian HF. However, electrical stimulation of the ventral pigeon HF was demonstrated to suppress plasma CORT titres more dramatically when performed at the caudal-most site explored than at more rostral points (Bouillé and Baylé, 1973), perhaps indicating that the caudal pole is more involved in negative feedback inhibition of the HPA-axis. A greater role of the caudal avian HF in affective modulation is also consistent with the selective sensitivity of AHN in this region to UCMS in laying hens (Gualtieri *et al.*, 2019).

Rates of AHN also vary across the rostro-caudal axis of the HF in various avian species (Smulders, 2017). Levels of proliferation are higher in the rostral VZ of marsh tits (Patel *et al.*, 1997b), while rates of neurogenesis are higher in the rostral HF of black-capped chickadees than at the caudal pole (Barnea and Nottebohm, 1994). However, this effect may be season-specific, as this species typically has a higher neuron density in the caudal HF, while the increase preceding hoarding season occurs specifically in the rostral third of the structure (Smulders *et al.*, 1995). Expression of immediate early gene ZENK also indicates activation of more numerous neurons in the rostral than in the caudal HF whilst these birds fly around a room to forage, hoard and/or retrieve food (Smulders and DeVoogd, 2000). Turnover of new neurons is faster in the rostral chickadee HF, which exhibits a relatively higher rate of disappearance of thymidine-labelled cells over survival times longer than six weeks (Barnea and Nottebohm, 1994). These collective findings point to a particular contribution of adult-born neurons in the rostral HF to spatial processing and memory, as modulated by the demands of hoarding behaviour. However, species differences are apparent, as in the non-hoarding zebra finch, more new neurons have been recorded in the caudal than in the rostral parts of the hippocampal formation (Barnea *et al.*, 2006). Additionally, in brown-headed cowbirds (*Molothrus ater*) and red-winged blackbirds (*Agelaius phoeniceus*) the caudal HF exhibits a higher density of DCX staining and more DCX^+ round and fusiform cells (Guigueno *et al.*, 2016), in the ventral but not the dorsomedial area (Smulders, 2017). A tendency for a greater density of thymidine-labelled neurons has also been observed in the caudal than in the rostral part of HF for captive mountain chickadees (Pravosudov and Omanska, 2005). Overall, therefore, there is some evidence that structural and functional differences exist across the longitudinal (rostro-caudal) axis of the avian HF, though these may vary between species. Both neuroanatomy and certain experimental findings suggest that the caudal subregion of the avian HF resembles the temporal

mammalian hippocampus, in terms of a greater role in affective regulation (Bouillé and Baylé, 1973) and enhanced sensitivity to stress (Gualtieri *et al*., 2019).

1.7 Project aims & hypotheses

Based upon the reviewed literature, the process of AHN appears to present a promising candidate for an integrated biomarker of experience in non-verbal animals. AHN correlates with self-reported subjective emotional experience in humans and is mediated according to the valence of long-term experiences in rodent studies, in parallel with behavioural indicators of affective state. Evidence in avian species further suggests that responses of adult neurogenesis to chronic stress and certain positive experiences are conserved in the HF of birds. With relation to the possibility of assessing welfare in commercial poultry, the sensitivity of AHN in domestic chickens to UCMS (Gualtieri *et al.*, 2019) and chronic food restriction (Robertson *et al.*, 2017) has been demonstrated.

1.7.1 Hypotheses

It is hypothesised that AHN in the laying hen HF will be modulated by those long-term conditions and experiences that are salient enough to induce alterations in the affective state of hens, in a direction dependent upon their valence. Conditions or experiences that are either prolonged or repeated and of notable detriment to welfare will induce a depressive-like state of chronic stress in hens, indicated by a relative suppression of neurogenesis in the HF. In contrast, conditions or experiences that are positive, or enriching, will be associated with elevated levels of AHN.

It is further posited that AHN across the septo-temporal axis of the avian HF may exhibit differential sensitivity to certain experiences, in a manner mirroring the mammalian HF. As the rostral avian HF connects to the septum, whilst the caudal subregion appears to exert greater regulatory influence over the HPA axis (Bouillé and Baylé, 1973), the latter region is hypothesised to be homologous to the temporal rodent hippocampus, which exhibits preferential sensitivity to certain sources of stress (see section 1.5.2). The suppression of AHN induced by chronically stressful conditions is thus predicted to be more pronounced in the caudal region of the laying hen HF. Observations in food hoarding birds further indicate that AHN is stimulated to a greater extent in response to spatial memory demands within the rostral HF (e.g. Barnea and Nottebohm, 1994; Smulders and DeVoogd, 2000), leading to the prediction that the exposure of hens to complex and/or enriched environments might recruit relatively more new neurons to this subregion.

1.7.2 Aims

Broadly, the aim of this project is to quantify endogenous markers of AHN in select samples of laying hens post-mortem, in order to provide information regarding the comparative long-term experience of larger groups of birds. As AHN in the mammalian and avian brain co-varies with prolonged experiences in a valence-specific manner, its quantification in the HF of laying hens with experiences that are known; be it longitudinally measured or experimentally controlled, will indicate which of these are important and/or salient enough to have a significant (positive or negative) impact on welfare. These findings may highlight areas wherein targeted welfare interventions would improve the long-term affective state of commercial hens. Furthermore, post-mortem quantification of AHN may facilitate comparison of the relative degree of chronic stress associated with differing housing systems or husbandry practices, as well as evaluation of the efficacy of interventions designed to enhance welfare, such as provision of access to an outdoor range.

In order to assess the welfare implications of commercially relevant experiences for laying hens, markers of AHN will be quantified in samples of birds, wherein *i*) aspects of the housing environment are experimentally manipulated, *ii*) individual use of the outdoor range is longitudinally tracked, and *iii*) severity of naturally-occurring keel bone fractures in a multi-tier housing system is longitudinally monitored. Finally, hens of varying physical condition will be sampled from a caged and a non-caged commercial housing system managed by the same production company, in order to assess differences in associated cumulative experience reflected by relative levels of AHN. In all studies, AHN in the rostral and caudal HF will be measured separately to determine whether sensitivity to various experiences indeed differs across this axis. In addition to addressing the aforementioned hypotheses, these explorations will further indicate whether individual differences in long-term experience predict AHN levels in the chicken brain in a linear manner.

Quantification of AHN will focus upon the measurement of endogenous marker of immature neuronal differentiation DCX, given that this method does not require *in vivo* administration and is well validated in both rodents (Couillard-Despres *et al.*, 2005) and birds (Balthazart *et al.*, 2008). However, we also aim to explore the potential use of molecular biology to more rapidly quantify the expression of a variety of candidate genetic markers of stressful experience in the HF, relating to neurogenesis and CORT regulation (e.g. MR and GR).

1.8 References

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Chapter 2. Expression of Genetic Markers of AHN Following Long-Term Housing in Experimental Conditions Designed to be Preferred or Non-Preferred

2.1 Abstract

Design of the housing environment and resources provided may influence the long-term welfare of commercial laying hens. Accessing nest boxes, perches and foraging materials appears to be a behavioural priority for hens, but whether the quality of each alternative is important to their overall experience is unclear. For this reason, hens were housed in an environment that was designed to be "Preferred" (P; n = 15) or "Non-Preferred" (NP; n = 15) for 26 weeks. P pens were large and had deep wood shavings as litter, while NP pens were smaller and had a wire floor. P pens contained more expansive nest boxes and perches than those in the NP pens, along with a peat/sand mix dust bath which was absent from the NP housing. P hens were also given daily positive reinforcers (e.g. food treats), while NP hens experienced punishers (e.g. water spray). At the end of the housing period, a range of physiological and behavioural measures were taken, and birds were given a series of two-way preference tests between their experimental housing (either P or NP) and a housing environment intermediate to both conditions. Tissue was collected from the HF, and the expression of mRNA transcripts for a range of candidate genes relating to adult hippocampal neurogenesis (AHN) and glucocorticoid regulation was measured using quantitative PCR. Preference for the experimental housing relative to the intermediate conditions was significantly higher for birds housed in P than in NP conditions. However, no other measure differentiated between hens housed in the two conditions. A minority of birds from each condition made the unexpected choice, and serum corticosterone was higher in these individuals than in hens that made the majority choice. In the caudal HF, expression of doublecortin (*DCX*) mRNA was also lower in hens that made the minority choice. The *MR*/*GR* mRNA ratio was lower in the rostral HF of NP housed hens that made the minority choice (in favour of the NP conditions), which might suggest that this subgroup of birds was the most stressed. Expression of proliferative marker *PCNA* was higher in hens that chose the intermediate conditions over either experimental housing condition. The validity of transcription as a proxy for AHN in chickens is currently uncertain, and consistency between qPCR assays was lower when cDNA came from different reverse transcriptions. However, the existing and novel measures of experience both suggest that variation between individuals, reflected by their preferences, may exceed differences arising from an inanimate housing environment designed to be relatively positive or negative for welfare.

The dataset is stored in a publicly accessible repository, at:
https://doi.org/10.25405/data.ncl.14140697.

2.2 Introduction

In attempts to enhance the welfare of commercial laying hens alongside production, much attention has been given to the design of the housing environment and its furniture. Modern commercial housing systems typically include several inanimate components that may be considered forms of enrichment when compared to conventional cages. Perches and nest boxes are generally provided, while systems may also contain further additions such as scratch mats, dust bathing areas, and foraging materials. The welfare implications of such provisions may be difficult to assess on-farm, as components are generally not provided in isolation, and multiple other variables (e.g. group size, health, etc.) may influence the cumulative experience of the hens. Furthermore, while the presence of certain components may be associated with beneficial outcomes in comparison to their absence, providing different forms of each item (e.g. enclosed versus open nest boxes, single- versus double-tiered perches, dust baths with different substrates) is likely to be associated with varying financial costs and husbandry requirements, but whether these variants are perceived to have different value by the hens, and thus have differing consequences for welfare, is often unclear.

Previous research has used tests of decision making to determine aspects of the housing environment that are important from the perspective of the hen. This approach assumes that even domesticated animals in artificial settings are capable of integrating relevant sensory inputs and selecting the option best suited to their own interests (Nicol *et al.*, 2009). As such, studies have examined which features or conditions chickens exhibit a significant preference for in controlled choice tests, and which they will work to obtain (or avoid). Collectively, this information may facilitate the design of experimental conditions that are relatively positive and negative from the perspective of the majority of hens, by collating the preferred/sought-after and non-preferred/avoided variants of each housing aspect or component, respectively.

In terms of floor substrate, research indicates that laying hens prefer shavings to wire floors (Weeks and Nicol, 2006), and while it has been argued that substrate preference may depend upon experience during rearing, birds reared on wire were found to switch their preference to shavings after a short period of exposure to this substrate (Nicol *et al.*, 2001). Foraging appears to be a behavioural priority for hens (Weeks and Nicol, 2006), and this behaviour is facilitated by the provision of litter. While imposing a "cost" of squeezing through a narrow entrance to access pens containing various resources reduced the overall number of pen entries and the frequency of other behaviours, it did not alter the amount of time that hens spent pecking and scratching in woodchip litter (Bubier, 1996a). Hens allocated around 38% of their time budget

during a nine hour photoperiod to pecking, scratching and feeding, and this figure was not altered by the addition or absence of feed in the litter (Bubier, 1996b). Both absence of substrate for foraging and administration of corticosterone (CORT) cause an increase in feather pecking (El-lethey *et al.*, 2001), suggesting that the re-direction of foraging behaviour towards conspecifics is associated with stress. Provision of foraging material in the form of long-cut straw has been associated with lower rates of feather pecking, higher egg production, shorter tonic immobility (TI) and lower heterophil:lymphocyte (H:L) ratios, compared to pens not provided with straw (El-Lethey *et al.*, 2000). If opportunity to enact naturalistic foraging behaviour is indeed important for welfare, a housing environment containing (deep) litter would be predicted to be superior to an environment with a wire floor.

Research also indicates that hens value nest boxes, perches and dust baths as components of housing systems. In particular, hens place a high value on access to discrete, enclosed nest sites, and the behavioural priority ascribed increases with proximity to the time of egg-laying (Cooper and Appleby, 2003; Weeks and Nicol, 2006). Hens are willing to pay high "costs" to gain access to nest boxes before laying, including squeezing through narrow gaps (Bubier, 1996a) and pushing to open resistance-loaded doors (Cooper and Appleby, 2003). Indeed, in order to access a nest box close to the time of laying, hens would squeeze through an extremely narrow gap, with a width that was not attempted to access food until hens had been deprived for an average of eight hours (Cooper and Appleby, 1996a). Two-way preference tests indicate that hens select strongly in favour of enclosed nests, at the expense of more naturalistic exposed nests (Appleby and McRae, 1986). Presence of a perch in front of the next box was also found to increase the proportion of eggs that hens laid within it (Cronin *et al.*, 2005), as the perch was used for access.

Where perches are provided, up to 100% occupancy is reported at night, with most hens using the highest perches available (Blokhuis, 1984; Olsson and Keeling, 2000). Where perches are not provided, hens choose instead to roost on the highest accessible fixtures and fittings (Appleby *et al.*, 1998). Perches are also used to an extent during the day (Weeks and Nicol, 2006), and willingness of hens to access them was not hindered by the cost of squeezing through a narrow gap (Bubier, 1996a). Hens are willing to push through resistance-loaded doors to access perches at night, and opened significantly heavier doors to gain access to a perch than to a "sham perch" (which could not actually be used for perching) (Olsson and Keeling, 2002). When perches were removed from pens, hens spent less time sitting and more time moving than when perches were available, and when the perches were visible but blocked by a clear barrier, hens exhibited more attempts to take off (Olsson and Keeling, 2000). These behavioural findings were interpreted as expressions of frustration combined with increased exploration to

find an alternative roosting site. Beyond height, hens appear to show little preference for other properties of perches, such as width and angle (Appleby *et al.*, 1998).

Birds with continual access to litter have been reported to dustbathe for an average of one 27-minute bout per day (Vestergaard, 1982). In cage systems that do not provide a litter substrate, hens are observed to engage in bouts of sham dustbathing on the wire floors (Hughes and Duncan, 1988). Such abnormal behaviours are considered to represent the nearest approximation of a behavioural priority that cannot be adequately fulfilled (Weeks and Nicol, 2006). When provided with a suitable litter substrate, hens that were previously deprived of such materials spend a longer time dustbathing (Vestergaard, 1982), which suggests that sham dustbathing is not sufficient to satisfy the motivation of hens to dustbathe. Several studies have found that peat and sand are preferred over substrates such as sawdust and straw for the purpose of dustbathing (Petherick and Duncan, 1988; van Liere and Siard, 1991; De Jong *et al.*, 2007). These preferences may be associated with the efficacy of bathing in each substrate for plumage maintenance, as hens housed with peat had the fluffiest feathers, lowest feather lipids and lowest back temperature (indicative of a high capacity for thermo-insulation) (van Liere and Siard, 1991).

Finally, the motivation of hens to access additional areas of space has been assessed in studies with hens trained to key-peck. This work has indicated that a cage size of 400cm^2 per bird is sufficient most of the time, but for up to a quarter of the day, hens will work to obtain a cage size of up to 1500cm^2 per bird (or 6000cm^2 between four birds) (Faure, 1986). A subsequent study found that hens would work to spend around half the day in cage floor areas greater than 1800cm^2 (between four hens), and that the number of key pecks for access to wire- or litter-floored cages did not differ (Lagadic and Faure, 1987). The available space thus appeared to be a high behavioural priority, which at least sometimes exceeded access to litter (Weeks and Nicol, 2006).

Based upon these findings, the present study sought to compare the long-term experience of hens housed in conditions that were designed to be either “Preferred” (P) or “Non-Preferred” (NP) by the majority of birds. Compared to NP pens, the P pens were larger and had deep shavings on the floor in place of wire. The NP pens contained a low, single-tiered perch and a roofed nest box with wire sides and no furnishings, whereas the P pens contained high, double-tiered perches, a furnished nest box with wooden sides and a perch at the front, and a dust bath with a mix of sand and peat substrate (no dust bath was provided in the NP pens). In addition, the P housing conditions were accompanied by the daily provision of positive reinforcers, in

the form of treat foods or a heated pad, while the NP housing conditions were accompanied by daily punishers, which took the form of water spray, presentation of a salient novel object, and playback of conspecific alarm calls or dog barks. The latter group of stimuli could be argued to mirror the mild, semi-unpredictable paradigms of chronic stress employed in rodent models, which are associated with suppressions in adult hippocampal neurogenesis (AHN) (e.g. Surget *et al.*, 2011; Hawley *et al.*, 2012; Tanti *et al.*, 2012). While chronic stress also appears to be associated with the downregulation of AHN in avian species (Barnea and Nottebohm, 1994; LaDage *et al.*, 2010; Robertson *et al.*, 2017; Gualtieri *et al.*, 2019), a stimulatory influence of experiences with positive valence, beyond spatial memory challenge (e.g. LaDage *et al.*, 2010), has yet to be demonstrated in birds. A previous study found that pigeons housed in enriched cages exhibited an increased number of DCX-expressing immature neurons (Melleu *et al.*, 2016), but this group also displayed longer durations of tonic immobility, making it unclear if the enrichment provided was a positive affective experience for the birds. Whilst providing laying hens with adequate resources to fulfill their behavioural needs may prevent the experience of chronic stress, positive affective experiences are also important for welfare (Lawrence *et al.*, 2019). Pharmacological manipulation has demonstrated that treat foods (e.g. mealworms) activate neural circuits involved in the subjective hedonistic experience of reward, as anticipatory behaviour is reduced in hens administered an opioid receptor antagonist (Taylor *et al.*, 2020). As such, the inclusion of rewards was designed to ensure that the P conditions both minimized stress and offered positive experiences, while the NP conditions included potential inanimate and experiential sources of stress.

It is also important to consider the notable individual differences highlighted by research into the preferences of laying hens, whereby a minority of hens make choices different to those made on average (Weeks and Nicol, 2006). For example, though hens generally prefer enclosed nest boxes (Appleby and McRae, 1986), a minority (~19%) of birds in one study of motivation consistently preferred to lay in litter trays, and were equally willing to exert themselves by pushing high resistance doors in order to gain access to this type of nest (Kruschwitz *et al.*, 2008). Similarly, a minority of hens were willing to work hard for access to a second peat dust bath, while the majority pushed more weight for this access when deprived of a dust bath in their pen (Widowski and Duncan, 2000). In commercial systems providing enclosed nest boxes, some hens always tend to lay on the floor. These floor layers perform more "nest-seeking" locomotor behaviour (Cooper and Appleby, 1996b) and may not perceive the nest box alternatives provided as satisfactory (Guesdon and Faure, 2004; Weeks and Nicol, 2006). Social factors may also interact with priorities, as half of tested hens would no longer work a push-

door to access a perch when another bird was using it, or roosting on the adjacent floor (Olsson and Keeling, 2002). To improve welfare for the flock, housing environments must almost always be designed in accordance with the preference of the majority, which may make them less well suited to the needs of the minority. It is assumed that hens can form associations between conditions and their experience in them (Nicol *et al.*, 2009). Therefore, hens that do not select an environment that they have lived in during a preference test might be inferred to have had a relatively poorer experience during that time, which may be reflected by other, sensitive measures of chronic stress.

As such, following long-term (26 week) housing in the "Preferred" or "Non-Preferred" conditions, individual preference for the experimental housing experienced versus an intermediate environment was measured in a series of two-way choice tests. Several physiological parameters associated with chronic stress were measured (serum CORT, H:L ratio, etc.), in addition to a behavioural measure of anhedonia in the form of mealworm consumption (akin to sucrose consumption in rodents). The results of previous studies suggest that certain traditional measures do not reflect the behavioural priorities of birds, for example as neither the absence of a nest box nor the sudden denial of access to one was associated with CORT levels in plasma or egg albumen (Cronin *et al.*, 2013). Quantification of AHN may provide a more sensitive measure capable of integrating positive and negative experiences on a cumulative basis (see section 1.4.6), but morphological methods to quantify adult-born cells are time consuming, which may limit application to welfare questions. Quantification of mRNA using quantitative real-time PCR (qPCR) is more rapid, and our research group has previously established that transcription of the *DCX* gene in the mouse hippocampus reflects DCX-immunoreactive cell densities under control and enriched housing conditions (Gualtieri *et al.*, 2017). Mechanisms by which stress-induced CORT downregulates AHN appear to causally involve glucocorticoid receptors (Oomen *et al.*, 2007), and the ratio of mineralocorticoid receptors (MR) to glucocorticoid receptors (GR) is altered by prolonged stress in mammals (Webster *et al*., 2002; Zhe *et al*., 2008; Klok *et al*., 2011) and birds (Dickens *et al*., 2009; Banerjee *et al*., 2011). It is also more common for MR and GR expression to be quantified in terms of mRNA abundance (e.g. Herman, 1993; Klok *et al.*, 2011). As an exploration of this methodological alternative, the expression of mRNA transcripts for candidate genetic markers of AHN (*DCX*, *PCNA* & *DCLK1*) and glucocorticoid negative feedback (*MR* & *GR*) was therefore quantified in the HF. The association of the established and novel measures taken with both housing condition and individual choice was explored, with the aim of ascertaining *i)* whether the differences in design of the housing environments, combined with discrete

positive reinforcers or punishers, were salient enough to impact the cumulative affective experience of hens, and *ii)* whether these external conditions interacted with individual preferences to determine the relative stress experience.

2.3 Methods

The following experiment was conducted at the School of Veterinary Sciences, Bristol University, by a team led by Dr. Liz Paul, Prof. Christine Nicol and Prof. Mike Mendl. This group was responsible for the experimental design, husbandry and the collection of behavioural and physiological data. Hippocampal tissue collection (subsequent to Schedule 1 dispatch), processing of samples for molecular biology, quantification of mRNA and the reported statistical analyses were conducted as part of the submitted doctoral research project.

2.3.1 Ethical statement

Work was approved by the University of Bristol Animal Welfare and Ethical Review Body and conducted under U.K. Home Office Licences (PPL: 30/2779 and 30/3392). Animal use and care was in accordance with the Animals (Scientific Procedures) Act 1986, EU directive 2010/63/EU and the UK Home Office code of practice for the housing and care of animals bred, supplied or used for scientific purposes. Collection and post-mortem analysis of tissue was approved by the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549).

2.3.2 Animals & husbandry

Subject were 30 medium-brown commercial laying hens, sourced from a commercial organic breeder at 18 weeks of age. On arrival at the experimental farm, hens were immediately housed in pens of five birds, and remained in these groups for the duration of the study. All birds were wormed, treated for mites and individually marked using coloured leg bands and a patch of coloured stock marker spray paint (Super Sprayline, Ritchey, Ripon, U.K.) on their tails. Throughout the study, all birds received the same *ad libitum* feeding regime (Farmgate Layers' Mash, BOCM Pauls, Ipswich, Suffolk, U.K.). They were kept on a 12-hour light-dark cycle (light period 07:00–19:00 hours) under fluorescent lighting (150 lux), with an ambient temperature of 19–21°C. General background noise on the farm was low (max 50Db). Eggs were collected daily, pens were cleaned weekly, and the health and wellbeing of birds was regularly monitored by professional caretakers.

Initially, birds were housed in baseline living conditions for a period of 10 weeks, followed by an additional two weeks during baseline data collection (results to be reported elsewhere). All pens used in the study were 1.8 m tall, with concrete walls to the back and chicken wire fencing and door to the front. The baseline pens were 2.25 m long and 1.22 m wide, with 20mm deep wood shavings on the floor. They also contained a single-tier perch (1 m wide and 150 mm high) a dustbath filled with wood shavings (0.8 x 1.22 m) and a double-tiered roofed nest box (300 x 300 x 830mm) that had solid wooden sides but no furnishings. These baseline conditions were designed to be intermediate to the two housing conditions employed in the experimental phase.

2.3.3 Housing manipulation

At week 12, three pens of five birds were allocated to the "preferred" (positive long-term valence) treatment group, and the other three pens were allocated to the "non-preferred" (negative long-term valence) group, so that there were 15 hens in each condition. At this point, each home (baseline) pen was modified in terms of its size and pen furniture to become a 'treatment' pen. As explained in the introduction, all features of the "preferred" (P) housing condition (e.g. size of pen, floor substrate, presence and size of perches, presence of dustbath, etc.) were designed on the basis of experimental evidence from previous studies which indicated that chickens will work to obtain them, or show a significant preference for them (reviewed in Weeks and Nicol, 2006). All features of the "non-preferred" (NP) housing condition were based on experimental evidence indicating that chickens avoid them in preference tests, and/or will work to avoid them in consumer-demand tests.

The P pens were 3.06 m long and 1.22 m wide, with 100 mm deep wood shavings on the floor. They contained double-tier perches (1 m wide and 300/600 mm high), a dustbath filled with a peat substitute/sand mix (0.8 x 1.22m), and a double-tiered, roofed nest box (300 x 300 x 830 mm) with wooden sides, rubber matting bases, a perch at the front and backing made from artificial grass. In contrast, NP pens were 1.52 m long and 1.22 m wide and had a wire floor. They contained a single-tier perch that was 400mm wide and 50mm high, no dustbath, and a single-tier, roofed nest box (300 x 300 x 830 mm) with wire sides and no furnishings. The P pens had an area of shade above the perch, but NP pens (and baseline/intermediate pens) did not. Hens in NP pens were exposed to taped white noise (80dB) for four hours per day. Hens in the P housing condition received a selection of discrete rewards four times per day, consisting of a heated pad and 50g portions of sweetcorn, spaghetti, or chopped cheese. Birds in the NP housing condition received discrete punishers four times per day. These constituted a small

plastic inflatable toy being lowered into the pen from above, taped conspecific alarm calls, taped dog barks, and water spray.

2.3.4 Behavioural & physiological data collection

After birds had completed a total of 26 weeks in either the P or NP housing conditions, behavioural and physiological data was collected during an additional period of two weeks, wherein hens remained in their experimental pens. As part of a physical examination (with other measures reported elsewhere), birds were weighed. On a separate occasion, blood samples were collected from each hen within two minutes of capture. 4 ml was taken from a wing vein using a 23-gauge 16 mm needle, divided between a Serum Collection Vacutainer Tube and an EDTA Blood Collection Vacutainer Tube (BD Vacutainer®) and placed immediately on ice. Two blood smears were made on glass microscope slides, and counts of heterophils (H) and lymphocytes (L) were quantified and used to calculate the H:L ratio (Gross and Siegel, 1983). Blood samples collected for serum extraction were left on ice overnight, before being centrifuged at 1200 rpm for 15 min. Serum was stored at -18°C prior to external analysis of CORT concentrations (Cambridge Specialist Laboratories). Samples of whole blood were analysed for levels of glucose (Langford Diagnostics Laboratories). Blood glucose levels are a downstream result of changes in CORT, which were previously associated with individual differences in choice for housing environments in laying hens (Nicol *et al.*, 2009). DCX^+ cell densities were previously found to correlate positively with blood glucose levels in adult bonnet macaques (Perera *et al.*, 2011).

Given that mealworms are a highly valued food item for hens, measuring their consumption may provide a behavioural index analogous to sucrose consumption in rats and mice, which indicates whether individuals are experiencing anhedonia. For the mealworm consumption test, each hen was placed on the floor of the testing room and given a ceramic bowl containing 100g of live mealworms. The mass of mealworms remaining after one minute was used to calculate the mass of mealworms consumed (g) per minute. Two tests were conducted on sequential days, and the mean mass consumed was calculated.

Following the collection of other data, all birds were given a series of T-maze choice preference tests, in order to determine whether their overall preference was for either an intermediate housing condition pen (resembling the one that they had experienced in the baseline phase), or a Preferred/Non-preferred living condition pen (resembling the one they had experienced during the experimental phase). Two appropriately furnished test pens (baseline and P or NP condition) containing food and water were set up opposite one another. Each group of five pen-

mates was housed in the baseline pen for the night preceding testing. Approximately 10 minutes before testing commenced, the five hens were placed together in a poultry transport crate. All faeces and eggs were removed from the baseline pen, food and water were refreshed, and a connecting T-maze tunnel was placed between the two pens. Birds were tested individually, in a predetermined random order and using a predetermined, randomly assigned starting-box position. Initially, just one of the two sliding doors at the tunnel exits was opened at a time, requiring each bird to make two forced choices: one to the baseline and one to the P or NP pen. After a pen was entered during one of these forced choices, the hen was confined within it for two minutes. Immediately after both forced choices had been made, each hen was allowed to make three free choices, each of which was followed by a two-minute confinement period. Once all five hens had been tested, the protocol was repeated in the same order to allow a further four free choices per bird. If a hen failed to make a choice within two minutes of being placed in the starting box, they were removed from the apparatus and 'no choice' was recorded as the test result. The hen was then replaced within the starting box to begin the next test. As such, each bird underwent a total of seven two-way choice tests.

2.3.5 Tissue collection

Following collection of behavioural and physiological data, and after a total of 28 weeks of housing in the P or NP conditions, all birds were killed via an intravenous injection of pentobarbital. Immediately thereafter, brains were removed from the skull, placed into 0.1 M PBS in a Petri dish and divided along the longitudinal fissure with a scalpel. Hippocampi were dissected from both hemispheres and divided midway across the rostro-caudal axis to produce two subsamples per hemisphere (rostral & caudal) containing equal amounts of tissue, for separate processing. As the exact border between these putative functional subdivisions has yet to be clearly mapped out, this method constitutes an approximation of the boundary between the rostral and caudal HF. The four isolated hippocampal regions collected per hen were placed in individual sample tubes containing 1 ml of RNAlater® Stabilization Solution (Thermo Fisher Scientific, Loughborough, UK) and refrigerated for 24 hours before storage at -20°C.

Carcasses were examined post-mortem to assess aspects of body condition and internal organ masses. The body cavity fat, liver, and spleen were removed and weighed. Spleen mass was converted to a proportion of whole bird body mass for statistical analysis. In laying hens, relative spleen weight is reduced by exogenous administration of ACTH (Mumma *et al.*, 2006) or CORT (Iyasere *et al.*, 2017), and by exposure to unpredictable chronic mild stress (Gualtieri *et al.*, 2019). Interestingly, studies in mice have also previously reported greater spleen mass in

animals housed in enriched environments than those kept in standard cages (Gurfein *et al.*, 2012; Gurfein *et al.*, 2014), which may be associated with an enhanced immune response.

2.3.6 RNA isolation & reverse transcription

Isolation of RNA from hippocampal tissue was conducted according to a TriSure reagent protocol (Bioline, London, UK). Isolated HF subregions were introduced to tubes containing Lysing Matrix D (MP Biomedicals, Cambridge, UK) and 1ml Tri-reagent. Samples were lysed for 50 seconds each in a FastPrep^{TM} machine (speed 5), before 200µl chloroform was added and mixed by 15 seconds of vigorous shaking. To facilitate phase separation, samples were centrifuged at 13,000 rpm for 15 minutes (4^{o}C). The clear aqueous phase (450µl) containing the RNA was removed with a pipette and transferred to a clean tube, without disturbing the interphase. An equal volume of 100% ethanol was added to each sample and mixed by shaking. Purification of the RNA product, combined with DNAse treatment, was conducted using the Zymo Direct-zol™ RNA MiniPrep Kit (Cambridge Bioscience, Cambridge, UK), according to manufacturer's instructions. During the elution step, 30µl of sterile distilled water was added directly to the membrane. Following a one minute delay, the column was centrifuged for 30 seconds, before the resultant eluate was reintroduced to the column and centrifuged again, to increase the concentration of the product. Concentrations were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). As the 120 hippocampal samples collected (left-rostral, left-caudal, right-rostral and right-caudal subsamples for each of the 30 birds) exceeded the capacity of a PCR machine, they were divided into two batches of 60 samples for reverse transcription. Each batch contained half of the birds from each housing condition, with all samples from the same bird processed together (i.e. the rostral and caudal HF from both hemispheres). The Tetro™ cDNA Synthesis Kit (Bioline, London, UK) was used to reverse transcribe 2µg RNA per sample, according to the manufacturer's instructions. For seven samples, the extracted RNA was not of sufficient concentration, and these were omitted from analysis.

2.3.7 Template production for standard curves

Gene-specific primers were designed using the NCBI primer-BLAST tool and sequences are displayed in Table 2.1. To produce each gene-specific template, a standard PCR was conducted with four 25 µl volume reactions. Each contained 12.5µl of 2x MyFi Mix (Bioline, London, UK), forward and reverse primers (400nM), 8.5µl of sterile distilled water, and 2µl of chicken hippocampal cDNA template. For electrophoresis, 20µl of the cDNA solution was added to a 2% agarose gel containing ethidium bromide (0.5µg/ml). Following excision of PCR products,

cDNA was extracted from the gel using the Qiagen MinElute™ 50 Kit, according to manufacturers' instructions. A final elution volume of 40µl water was employed. This produced gene-specific templates with concentrations ranging from 6.5 to 9.2 ng/µl, measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Loughborough, UK).

Table 2.1. Gene-specific primers used for qPCR.

Gene	Accession	Orientation	Location of primer in nucleotide sequence (base pairs)	Primer Sequence (5'- 3')	Product Length (base pairs)
DCX	NM_001305674.2	Forward	801 – 820	AAGACGGCCCATTCGTTTGA	166
		Reverse	947 - 966	ATTTTTCGGGACCACAGGCA	
LBR	NM_205342	Forward	1464 – 1486	GGTGTGGGTTCCATTTGTCTACA	80
		Reverse	1526 - 1543	CTGCAACCGGCCAAGAAA	
NeuN/ Rbfox3	XM_004946288.1	Forward	635 – 654	AGCAGCCCAAGAGGTTACAC	191
		Reverse	806 - 825	ATTCAGCTTCTCTCGTGCCC	
DCKL1	NM_001257257.1	Forward	2331 – 2350	TGGACCACGGGTTTACCATC	127
		Reverse	2438 - 2457	ATCCTGGTTGCGTCTTCGTC	
PCNA	NM_204170.2	Forward	283 – 302	CAATGCGGATACGTTGGCTC	192
		Reverse	455 - 474	ACAGCATCACCAATGTGGCT	
MR	NM_001159345.1	Forward	2889 – 2911	TTGCTCCAGACCTGATTT	64
		Reverse	2932 – 2952	TGGCACAGTTCAAACATTGCA	
GR	NM_001037826.1	Forward	2092 – 2114	TCGTGAAAAGAGAAGGGAACTCA	121
		Reverse	2191 - 2212	AAAAACGTCTGGAAGCAAAAGC	

2.3.8 Quantitative PCR

Quantitative real-time polymerase chain reaction (qPCR) assays were conducted with SYBR® green fluorophore, according to a standard curve protocol. Reactions (20µl) consisted of 10µl Sensifast SYBR No-ROX master mix (Bioline, London, UK), forward and reverse gene-specific primers (400nM), 3.4µl sterile distilled water and 5µl of cDNA template. The qPCR assays utilised a Bio-Rad model machine (Bio-Rad, California, USA) with a 96-well plate. Manufacturer's instructions were followed for three-step thermal cycling conditions (see Table

2.2). Samples were run in singlicate over the same two batches that were used for reverse transcriptions, alongside standard curves of five-fold serial dilutions, run in duplicate, and two no-template controls. Assays were analysed using CFX-Manager software (Bio-Rad, California, USA) to calculate the molar quantities of cDNA present in the experimental samples with reference to the standard dilutions. In line with the validation process for qPCR, all primers were confirmed to produce a single melting peak, indicating amplification of a specific product. Efficiencies of standard curves ranged from 98.6% to 124.3%, whilst R^2 estimates exceeded 0.997. As employed previously in chickens (Dunn *et al.*, 2013), the Lamin-B receptor (*LBR*) gene was used as a control gene for normalisation. *LBR* is a component of the inner nuclear membrane (Olins *et al.*, 2010), meaning that its expression should be similar in all hippocampal samples and unresponsive to experimental housing conditions. *LBR* was thus quantified in each cDNA sample for every batch of the reverse transcribed product, to control for technical variations in the efficacy of the reverse transcription procedure between samples.

Table 2.2. The three-step temperature cycling conditions employed for quantitative PCR runs.

Cycles	Temperature	Duration	Process
1	95°C	2min	Polymerase Activation
40	95°C	5s	Denaturation
	60°C	10s	Annealing
	72°C	15s	Extension (acquired at end of step)

Measured molar quantities of each gene were log(10)-transformed and normalised using the Standard Score (Z_i) within the two (reverse transcription/qPCR) batches. For analyses of the *MR/GR* ratio, the ratio of the molar mRNA values was calculated prior to log(10)-transformation and normalisation within batches.

2.3.9 Methodological validation

In order to assess the reliability of quantified molar mRNA values, repeat measurements were conducted in two further subsets of samples. Though variability between reverse transcription and qPCR batches was controlled for through use of the Standard Score (Z_i), it is possible that this factor might obscure subtle differences in gene expression related to housing system. RNA from all samples from the left hemisphere (n=60) was therefore reverse transcribed in a single batch. This sample set included a rostral and a caudal HF sample from every hen (15 x P and 15 x NP). With a single qPCR assay per gene, confirmatory measurements of *LBR*, *DCX*, *MR*

and *GR* mRNA were conducted. The remaining cDNA was used to quantify expression of doublecortin-like kinase 1 (*DCLK1*), a microtubule associated protein expressed in adult neurons with similar functions to DCX (Burgess and Reiner, 2002; Deuel *et al.*, 2006), and mature neuronal marker *NeuN*, an alternate housekeeping gene reflecting the population of mature neurons. Measured molar values were log(10)-transformed, but it was not necessary to use Standard Scores (Z_i).

Finally, to assess how variation between individual qPCR assays compared to that arising between reverse transcriptions, repeat qPCRs for *DCX* and *LBR* were conducted in single batches with the remaining cDNA from the original reverse transcriptions. Because the influence of stress arising from NP housing was predicted to preferentially suppress AHN in the caudal HF, the measurement of *DCX* and *LBR* mRNA was repeated in the 60 caudal subsamples (originating from the left and right HF of every hen). Additionally, because the environmental complexity associated with enriched P housing was predicted to particularly influence AHN in the rostral HF (e.g. Tanti *et al.*, 2012; Gualtieri *et al.*, 2017), expression of proliferative marker *PCNA* was quantified in the remaining cDNA from the rostral subsamples (from both hemispheres, for all hens), along with control gene *LBR*. As the cDNA employed had been reversed transcribed over two batches (with half of the samples in each), measured quantities were log(10)-transformed and normalised using the Standard Score (Z_i) within these reverse transcription batches. Data for samples with residual target gene expression (after controlling for *LBR* expression in simple linear regressions) that was over 2.5x the interquartile range outside of this range was removed, and the remaining residuals were normally distributed (Shapiro-Wilk > 0.05). For *MR*/*GR* expression, the same process was followed for the log(10)-transformed expression ratio values.

2.3.10 Data analysis

All analyses were conducted using IBM SPSS Statistics (v24). To compare the preference of P and NP-housed hens for their housing conditions, the overall choice made by each hen was determined based upon whether the experimental or baseline/intermediate housing was chosen on a higher proportion of the seven choice tests. A generalised linear model with a binary response (logit function) was employed to determine if this categorical preference for the experimental housing differed depending on whether the conditions were P or NP. The proportion of times that the home environment was chosen over the baseline environment by each hen over the seven preference tests was also compared between the two housing conditions in a linear mixed model (LMM), alongside pen as a random factor and housing condition (P/NP)

as a between-subject fixed factor. To explore whether physiological measures of welfare differed between P- and NP-housed hens, body mass, serum CORT, blood glucose, H:L ratio (log(10)-transformed), spleen mass (as a percentage of body mass) and the mass of mealworms consumed were each entered separately as dependent variables in LMMs of similar structure, but which also accounted for individual differences in choice. There were two possible ways to categorise hens according to the outcomes of the preference test. Firstly, hens could be coded according to whether they made the majority or minority choice, with the former equating to the choice in favour of the objectively "better" conditions presented in the preference test for birds from both housing systems. The majority choice for hens from P housing was for the P pen, while the majority choice for hens from NP housing was for the baseline/intermediate pen. The minority choice for P hens was for the baseline pen, while the minority choice for NP hens was for the NP pen. Initially, majority versus minority choice was entered as a fixed factor and in an interaction term with housing condition in each LMM. However, hens could also be categorised according to whether they chose either the experimental conditions that they had been living in, or the baseline/intermediate conditions (which they had not experienced since 14 weeks previously). In this case, P-housed hens that chose their experimental conditions were still those that made the majority choice, but NP-housed hens that chose their experimental conditions were those that made the minority choice. In instances where the initial model revealed an interaction between housing condition and majority/minority choice, choice was re-coded in the latter manner (experimental/baseline) and the model was run with this variable in place of housing and choice.

To analyse hippocampal gene expression, separate LMMs with unstructured covariance were conducted with expression of mRNA for *DCX* and the *MR/GR* ratio (standardised within processing batches) as the dependent variables. These models included individual (hen) and pen as random factors, and *LBR* expression in the same sample as a covariate. Hemisphere and HF subregion were entered as repeated (within-subject) fixed factors, and housing condition (P/NP) and choice (majority/minority) were entered as between-subject fixed factors. Full factorial interactions between these four variables were initially explored, but where higher order interactions were non-significant, these were systematically removed (beginning with the highest (4-way) level, and starting from the term furthest from significance within a level) as a means of model simplification. As before, choice was re-coded as experimental versus baseline in instances that housing condition interacted with majority/minority choice.

To explore whether individual differences in hippocampal gene expression and other physiological measures of welfare were related over all hens, serum CORT and blood glucose

concentrations, H:L ratios, relative spleen mass and the mass of mealworms consumed were separately entered as covariates in LMMs that accounted for individual (hen) and pen, and included full factorial interactions between hemisphere, HF subregion and the covariate of interest. Again, non-significant higher order interactions were systematically removed as a means of model simplification.

As measurements of *DCX* and *LBR* were repeated three times in subsets of the same samples, results were compared to assess inter-assay reliability. Pearson's bivariate correlations were calculated between corresponding residual *DCX* mRNA expression measurements, after controlling for LBR expression in the same batch of cDNA.

Measured mRNA expression in qPCR assays of samples from a single subregion (rostral *PCNA* & *LBR* and caudal *DCX* & *LBR*, standardised for reverse transcription batch) was also explored in LMMs with unstructured covariance. Again, these models included individual (hen) and pen as random factors, with *LBR* expression as a covariate. Hemisphere was entered as a repeated fixed factor, while housing condition and choice (majority/minority) were between-subject fixed factors. Factorial interactions were again included, with non-significant higher order interactions and interactions between housing and majority/minority choice treated as before. The association of rostral *PCNA* expression with other physiological measures was explored in the same manner as previously. In samples from the left hemisphere, processed within a single batch, the expression of *DCX* was explored relative to both *LBR* & *NeuN* in separate models. Expression of *DCLK1*, relative to *LBR*, and the *MR*/*GR* ratio were additionally analysed in LMMs with a similar structure to those run previously, but which included HF subregion as a fixed factor and in factorial interactions, rather than hemisphere.

This work constituted a preliminary assessment of whether the expression of any mRNA transcripts from a range of candidate markers reflected the long-term experience of different housing conditions, or was associated with individual differences in preference for these conditions, or in other physiological parameters. As such, a large number of tests were conducted. In order to account for this statistically, the Bonferroni correction was applied to related groups of analyses. The threshold for significance (α) was calculated according to the number of tests conducted for different genes quantified in the same cDNA, or the number of covariates explored in relation to the expression of a single gene.

2.4 Results

2.4.1 Behavioural & physiological measures of welfare

Of the 15 hens housed in P conditions, 11 chose their experimental housing over the baseline/intermediate housing in more than half of the choice tests at the end of the experiment. Only four NP hens chose their housing over the baseline conditions. There was a significant effect of housing condition on this binary majority choice ($\chi^2_1 = 6.00$, $p = 0.014$). Relative preference for the home environment, as a proportion of the seven choices made, was also higher in hens from P than from NP housing ($F_{1,28} = 16.20$, $p < 0.001$). Descriptive statistics for behavioural and physiological measures collected in hens from the two housing conditions are presented in Table 2.3.

Table 2.3. Descriptive statistics for behavioural and physiological data collected from hens after 26 weeks of housing in either preferred (P) or non-preferred (NP) conditions.

	Preferred Housing (n=15)			Non-Preferred Housing (n=15)		
	M	SD	Range	M	SD	Range
Relative home preference	0.75	0.24	0.33 - 1.00	0.33	0.32	0.00 - 1.00
Body mass (kg)	1.89	0.13	1.66 - 2.21	1.86	0.16	1.63 - 2.2
Serum CORT (ng/ml)	1.64	0.79	0.54 - 3.18	1.73	0.51	0.84 - 2.96
H:L ratio	0.96	0.45	0.43 - 1.78	0.95	0.39	0.45 - 1.78
Blood glucose (mmol/l)	12.91	0.79	11.60 – 14.10	13.01	0.95	11.70 – 14.80
Spleen mass (g)	2.17	0.56	1.30 - 3.40	2.09	0.66	1.30 - 3.90
Mealworms consumed (g)	14.68	5.81	2.95 - 23.80	13.96	3.46	6.50 - 22.55

As six physiological parameters were compared between hens housed in P and NP conditions, a Bonferroni-corrected threshold of $\alpha = 0.008$ was applied. Serum CORT concentrations did not differ between P- and NP- housed hens ($F_{1,26} = 0.03$, $p = 0.862$) but there was a main effect of choice ($F_{1,26} = 12.59$, $p = 0.001$). CORT was higher in hens from both housing conditions that made the minority choice (M = 2.29, SEM = 0.198) compared to hens that made the majority choice (M = 1.46, SEM = 0.119, Figure 2.1). There was no interaction between housing and choice ($F_{1,26} = 1.44$, $p = 0.241$). Blood glucose concentration did not differ with housing condition ($F_{1,5.7} = 0.004$, $p = 0.952$) or choice ($F_{1,25.7} = 0.95$, $p = 0.338$), and there was no interaction ($F_{1,25.7} = 0.20$, $p = 0.659$). The same was true for H:L ratios (housing: $F_{1,24.0} =$

0.88, $p = 0.358$; choice: $F_{1,24.0} = 0.98$, $p = 0.333$, housing*choice: $F_{1,24.0} = 3.41$, $p = 0.077$), body mass (housing: $F_{1,4.8} = 0.12$, $p = 0746$; choice: $F_{1,24.7} = 1.15$, $p = 0.294$, housing*choice: $F_{1,24.7} = 0.02$, $p = 0.890$) and the mass of mealworms consumed (housing: $F_{1,4.5} = 0.10$, $p = 0.768$; choice: $F_{1,24.2} = 0.30$, $p = 0.589$, housing*choice: $F_{1,24.2} = 0.01$, $p = 0.909$) As a percentage of body mass, spleen mass did not differ with housing ($F_{1,26.0} = 0.40$, $p = 0.533$) or majority/minority choice ($F_{1,26.0} = 0.23$, $p = 0.636$), and the interaction did not reach the threshold for significance ($F_{1,26.0} = 4.34$, $p = 0.047$).

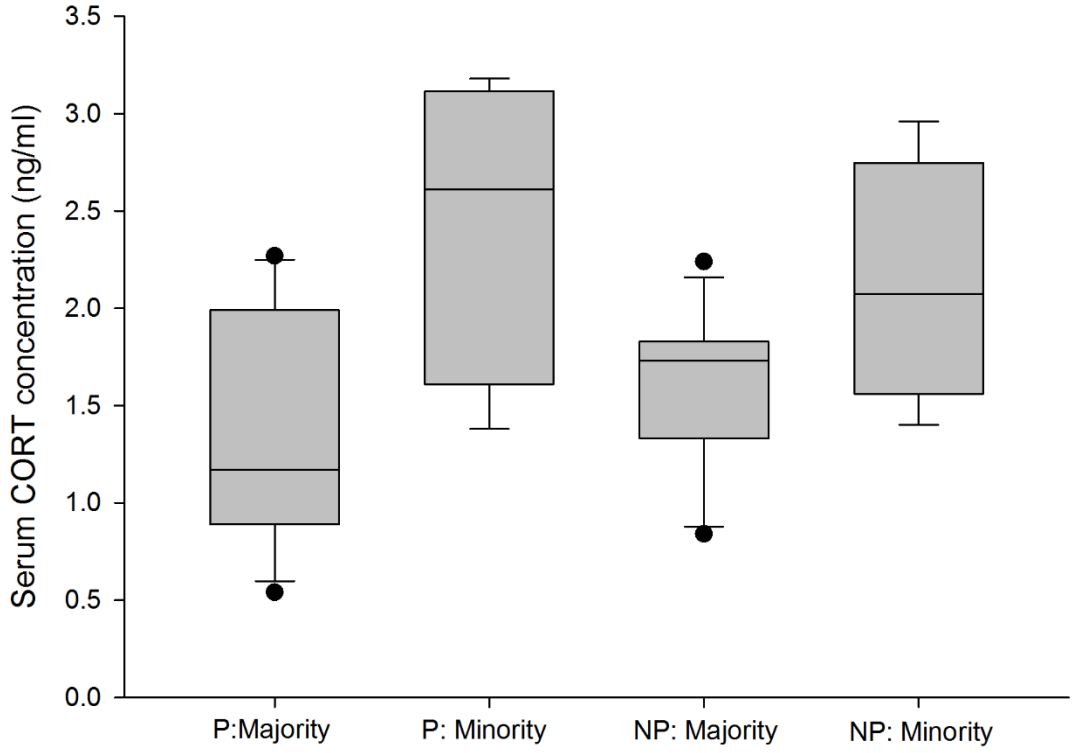

Figure 2.1. Serum CORT concentrations (ng/ml) of hens, grouped according to both housing condition (Preferred: P, or Non-Preferred: NP) and whether the overall choice made by the individual in two-way preference tests was for the option chosen by the majority of birds from that housing condition, or by a minority. P:Majority; P-housed hens that chose P conditions (n=11), P:Minority; P-housed hens that chose baseline conditions (n=4), NP:Majority; NP-housed hens that chose baseline conditions (n=11), NP:Minority; housed hens that chose NP conditions (n=4).

2.4.2 Control gene expression

Expression of control gene *LBR* did not differ between the HF subregions ($F_{1,22.4} = 0.11$, $p = 0.739$), or with housing ($F_{1,27.3} = 0.19$, $p = 0.670$), or choice ($F_{1,26.0} = 1.01$, $p = 0.324$). *LBR* expression was higher in samples from the left (M = 0.178, SEM = 0.088) than the right (M = -0.278, SEM = 0.197) hemisphere ($F_{1,21.2} = 6.61$, $p = 0.018$), and mean raw mRNA levels (standardised for processing batch) were similarly higher in the left hemisphere for all quantified genes. This effect was also significant for *MR* ($F_{1,20.9} = 5.98$, $p = 0.023$), and tended towards significance for *GR* ($F_{1,21.8} = 3.77$, $p = 0.065$; *DCX*: $F_{1,24.1} = 2.77$, $p = 0.109$). The effect

of hemisphere therefore likely arose from the (right) handedness of the experimenter who performed the HF dissections, and did not remain significant in models of target gene expression that accounted for *LBR* expression as a covariate (see below). There was no interaction between hemisphere and subregion on *LBR* expression ($F_{1,26.1} = 1.81$, $p = 0.190$), nor between hemisphere and housing ($F_{1,21.2} = 0.30$, $p = 0.587$) or hemisphere and choice ($F_{1,21.2} = 0.02$, $p = 0.889$). HF subregion also did not interact with housing ($F_{1,24.4} = 3.77$, $p = 0.064$) or choice ($F_{1,22.4} = 0.16$, $p = 0.691$), and there was no housing by choice interaction ($F_{1,25.5} = 1.15$, $p = 0.294$). While other three-way interactions were non-significant, the three-way interaction between hemisphere, subregion and housing reached significance ($F_{1,25.5} = 5.75$, $p = 0.024$). This finding can most likely be attributed to random differences in the efficacy of RNA extraction and reverse transcription in samples from different groups of hens, and highlights the necessity of accounting for such technical variation by controlling for *LBR* expression as a covariate in models of target gene expression. As expected, *LBR* expression significantly co-varied with target gene expression over all models ($F_{1,48.1} \geq 460.9$, $p < 0.001$).

2.4.3 Hippocampal gene expression (all samples)

Two tests were conducted to compare gene expression in all collected HF samples between P and NP housed hens that made the majority and minority choice. According to the Bonferroni correction, an α threshold of 0.025 was employed.

With *LBR* expression as a covariate, *DCX* expression did not differ between the hemispheres ($F_{1,21.0} = 0.62$, $p = 0.442$) or the HF subregions ($F_{1,19.8} = 0.36$, $p = 0.556$). There was no main effect of housing ($F_{1,25.2} = 1.32$, $p = 0.261$), or of choice ($F_{1,25.7} = 1.03$, $p = 0.319$), on *DCX* expression, nor did the factors interact ($F_{1,25.9} = 1.36$, $p = 0.255$). HF subregion interacted with housing ($F_{1,21.2} = 12.09$, $p = 0.002$). NP hens had higher expression of *DCX* in the rostral HF (M = 0.216, SEM = 0.171) than in the caudal subregion (M = -0.078, SEM = 0.143, $p = 0.012$). There was no subregional difference for P-housed hens ($p = 0.067$). *DCX* expression did not differ between P- and NP-housed hens within either the rostral ($p = 0.050$) or the caudal ($p = 0.965$) HF. Other two-way interactions were non-significant (subregion*choice: $F_{1,19.0} = 4.18$, $p = 0.055$; hemisphere*housing: $F_{1,21.3} = 2.56$, $p = 0.125$; hemisphere*choice: $F_{1,21.0} = 1.75$, $p = 0.201$; hemisphere*subregion: $F_{1,26.7} = 2.25$, $p = 0.146$). However, there was a three-way interaction between subregion, housing and choice ($F_{1,18.0} = 8.74$, $p = 0.008$). NP-housed hens that made the minority choice had higher *DCX* expression in the rostral HF than in the caudal subregion ($p = 0.002$). No other pairwise comparisons reached significance ($p \geq 0.034$). Other higher order interactions were non-significant and removed from the final model.

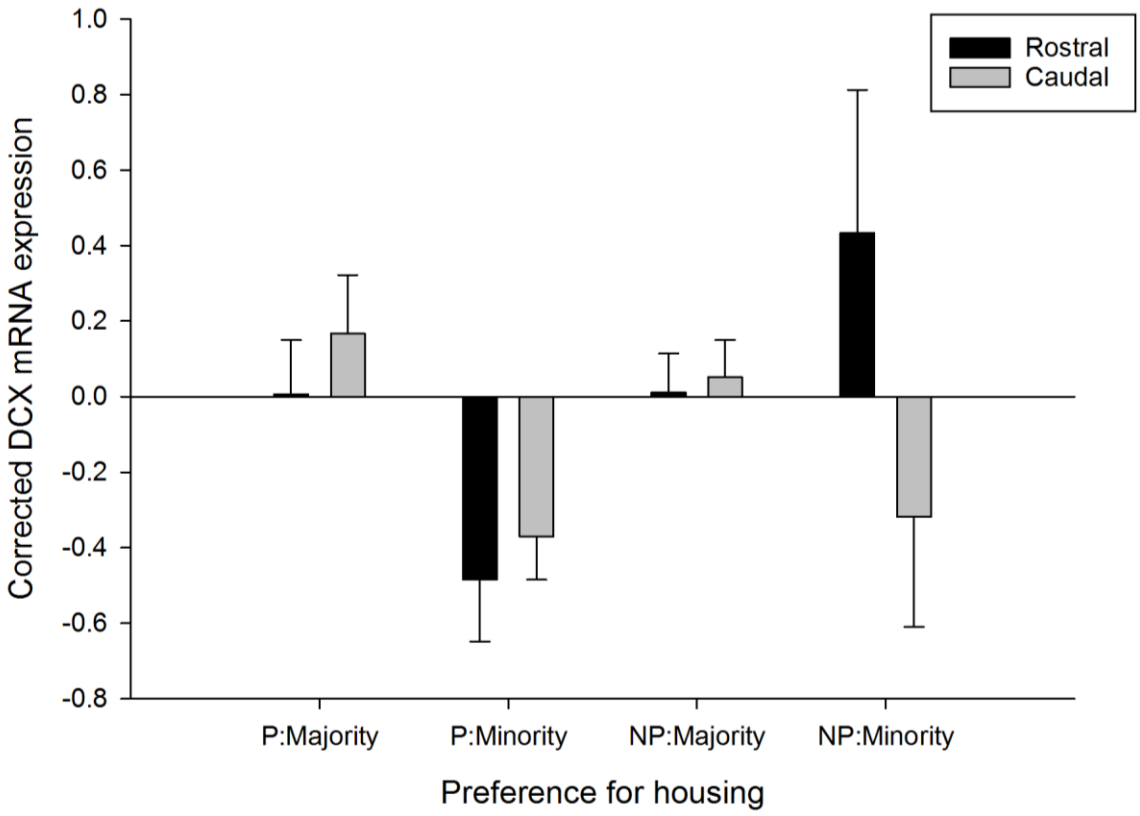

Figure 2.2. Mean expression of doublecortin (*DCX*) mRNA in the rostral and caudal HF subregions, grouped according to both housing condition (Preferred: P, or Non-Preferred: NP) and whether the overall choice made by the individual in two-way preference tests was for the option chosen by the majority of birds from that housing condition, or by a minority. P:Majority; P-housed hens that chose P conditions (n=11), P:Minority; P-housed hens that chose baseline conditions (n=4), NP:Majority; NP-housed hens that chose baseline conditions (n=11), NP:Minority; housed hens that chose NP conditions (n=4). Error bars represent +1 standard error. Gene expression values are residual after correcting for individual (hen), LBR expression, pen, and hemisphere, in LMMs.

The ratio of *MR* to *GR* receptor expression did not differ between the hemispheres ($F_{1,22.1}$ = 0.74, p = 0.400) or the HF subregions ($F_{1,21.8}$ = 3.38, p = 0.080; hemisphere*subregion: $F_{1,26.2}$ = 0.31, p = 0.585). There was no effect of housing condition ($F_{1,24.1}$ = 4.64, p = 0.041), or of choice ($F_{1,25.4}$ = 0.36, p = 0.555) on *MR*/*GR* expression, nor a housing by choice interaction ($F_{1,23.0}$ = 3.20, p = 0.087). Hemisphere did not interact with housing ($F_{1,22.3}$ = 0.15, p = 0.706) or choice ($F_{1,22.2}$ = 0.10, p = 0.750). Subregion also did not interact independently with housing ($F_{1,20.5}$ = 3.57, p = 0.073) or choice ($F_{1,20.5}$ = 4.11, p = 0.056), but there was a three-way interaction between subregion, housing and choice ($F_{1,21.1}$ = 6.20, p = 0.021, Figure 2.3). In the rostral HF, *MR*/*GR* ratio was lower for NP-housed hens that made the minority choice (M = -1.02, SEM = 0.431) than for both NP-housed hens that made the majority choice (M = 0.222, SEM = 0.247, p = 0.018) and for P-housed hens that made the minority choice (M = 0.645, SEM = 0.405, p = 0.008). NP-housed hens that made the majority choice had a higher *MR*/*GR*

expression ratio in the rostral HF (M = 0.222, SEM = 0.247) than in the caudal subregion (M = -0.354, SEM = 0.207, $p = 0.005$). There was a trend for P-housed hens that made the majority choice to similarly have a higher *MR*/*GR* ratio in the rostral HF (M = 0.253, SEM = 0.243) than the caudal subregion (M = -0.169, SEM = 0.202, $p = 0.030$). No other pairwise comparisons were significant. Other higher order interactions were non-significant and removed from the final model.

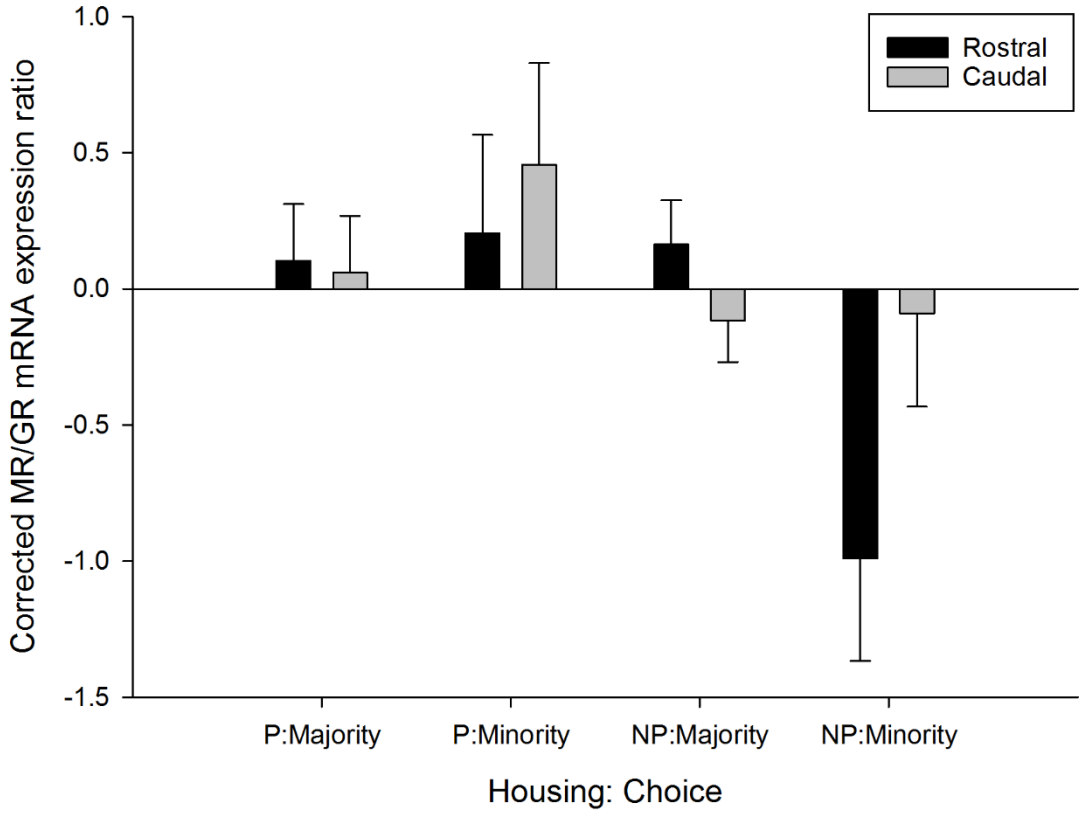

Figure 2.3. Mean expression ratio of mineralocorticoid receptor (MR) to glucocorticoid receptor (GR) mRNA in the rostral and caudal HF subregions, grouped according to both housing condition (Preferred: P, or Non-Preferred: NP) and whether the overall choice made by the individual in two-way preference tests was for the option chosen by the majority of birds from that housing condition, or by a minority. P:Majority; P-housed hens that chose P conditions (n=11), P:Minority; P-housed hens that chose baseline conditions (n=4), NP:Majority; NP-housed hens that chose baseline conditions (n=11), NP:Minority; housed hens that chose NP conditions (n=4). Error bars represent +1 standard error. Gene expression values are residual after correcting for individual (hen), LBR expression, pen, and hemisphere, in LMMs.

2.4.4 Relationship between gene expression & physiological welfare measures

Five physiological parameters were explored as covariates in relation to *DCX* mRNA expression in the HF. To account for this multiple testing, a threshold of $\alpha = 0.01$ was employed to detect significant associations. Individual differences in *DCX* expression within the HF were not predicted by individual differences in serum CORT ($F_{1,23.3} = 0.02$, $p = 0.891$), blood glucose ($F_{1,25.8} = 6.15$, $p = 0.020$), H:L ratio ($F_{1,24.1} = 1.90$, $p = 0.180$), or mealworm consumption ($F_{1,27.6}$

= 1.91, p = 0.179). Relative spleen mass did not predict *DCX* expression over the whole HF ($F_{1,29.4}$ = 0.45, p = 0.509), but interacted with subregion ($F_{1,20.2}$ = 8.42, p = 0.009). The slope of the relationship tended towards positive in the rostral HF (B = 2.169, SEM = 3.862, $F_{1,31.4}$ = 0.32, p = 0.578) and negative in the caudal HF (B = -5.323, SEM = 3.123, $F_{1,22.9}$ = 2.91, p = 0.102) but was not significantly different from zero in either subregion. There were no other interactions between the physiological covariates and HF subregion or hemisphere.

The same α level of 0.01 was applied to analyses relating *MR*/*GR* ratios in the HF to other physiological parameters. The *MR*/*GR* expression ratio was not predicted by serum CORT ($F_{1,19.6}$ = 6.91, p = 0.016), blood glucose ($F_{1,21.8}$ = 3.01, p = 0.097), H:L ratio ($F_{1,22.3}$ = 0.72, p = 0.406), or mealworm consumption ($F_{1,25.3}$ = 3.10, p = 0.091). Relative spleen mass did not predict MR/GR ratio over the whole HF ($F_{1,27.1}$ = 0.47, p = 0.499), but again interacted with subregion ($F_{1,22.7}$ = 8.68, p = 0.007). While the slope of the relationship was negative in the rostral HF (B = -8.514, SEM = 5.617, $F_{1,30.1}$ = 2.30, p = 0.140) and positive in the caudal HF (B = 0.098, SEM = 4.395, $F_{1,21.4}$ < 0.001, p = 0.982), neither slope differed from zero.

2.4.5 Methodological validation

Quantification of *DCX* and *LBR* mRNA was repeated in *1)* a single qPCR assay of all 60 caudal samples (employing cDNA from the previous reverse transcriptions) and *2)* a single qPCR of all 60 samples from the left hemisphere (with cDNA from a new reverse transcription). To assess the reliability of mRNA measurements, residual (standardised) *DCX* expression was compared in corresponding samples. Consistency between measurements from qPCR assays employing cDNA from the same reverse transcriptions was high (r(49) = 0.826, p < 0.001), despite the samples being processed over two separate qPCR assays in one case and in a single qPCR assay in the other. When using cDNA produced in different reverse transcription batches, measured *DCX* mRNA was significantly correlated (r(51) = 0.378, p = 0.005), but exhibited a weaker relationship.

2.4.6 Hippocampal gene expression in single batches

In samples from the caudal HF, *DCX* expression did not differ between the hemispheres ($F_{1,23.6}$ = 4.45, p = 0.241), or between hens housed in P versus NP conditions ($F_{1,3.5}$ = 0.001, p = 0.972). However, *DCX* expression was lower in individuals that made the minority choice (M = -0.291, SEM = 0.122, $F_{1,19.4}$ = 8.40, p = 0.009, Figure 2.4). Interactions were non-significant and thus removed from the final model.

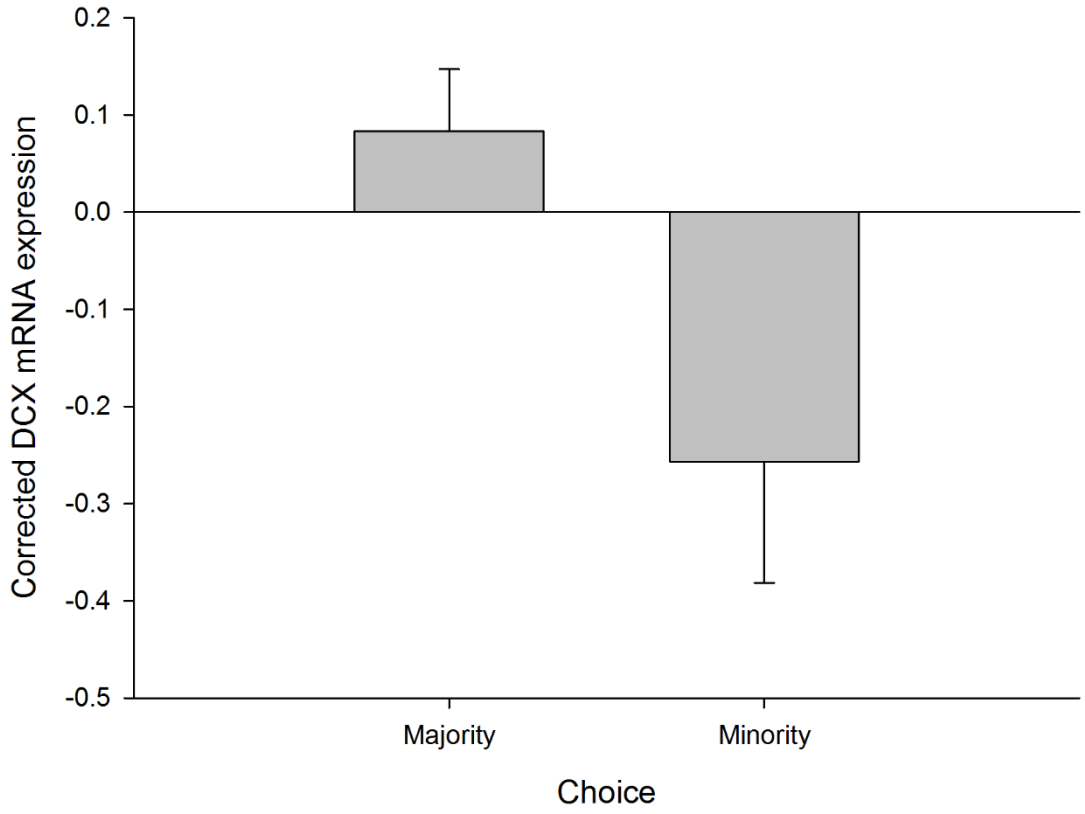

Figure 2.4. Mean expression of doublecortin (*DCX*) mRNA in a single batch of samples from the caudal HF, for hens that made the majority (n=22) or minority (n=8) overall choice in two-way preference tests. The majority choice was for the option designed to be the better of the two presented. Error bars represent +1 standard error. Gene expression values are residual after correcting for individual (hen), LBR expression, pen, and hemisphere, in LMMs.

Expression of proliferating cell nuclear antigen (*PCNA*) mRNA was quantified in a single batch of samples from the rostral HF. With *LBR* expression as a covariate, there was no main effect of hemisphere on *PCNA* expression ($F_{1,22.0} = 2.91$, $p = 0.102$), which also did not differ between the two housing conditions ($F_{1,5.2} = 3.41$, $p = 0.122$) or with choice ($F_{1,23.4} = 0.29$, $p = 0.597$). However, there was an interaction between housing and choice ($F_{1,23.5} = 5.29$, $p = 0.031$). As such, choice was re-coded as being in favour of either the experimental conditions the hen had recently lived in, or the baseline/intermediate conditions. There was a main effect of choice for experimental or baseline housing on *PCNA* expression ($F_{1,30.0} = 6.33$, $p = 0.017$), and this variable also interacted with hemisphere ($F_{1,25.9} = 4.81$, $p = 0.037$, Figure 2.5). *PCNA* expression was higher in hens that chose the baseline housing (M = 0.108, SEM = 0.077) than in hens that chose their experimental housing (M = -0.095, p = 0.075). This difference was specific to the left hemisphere ($p < 0.001$), with no difference present in the right hemisphere ($p = 0.496$). Hens that chose their experimental housing had lower *PCNA* expression in the right hemisphere than in the left hemisphere ($p = 0.004$). There was no hemispheric difference in *PCNA* expression for hens that chose the baseline housing ($p = 0.892$).

Again, α = 0.01 was the significance threshold employed for the association between the five physiological covariates of interest and HF gene expression. Individual differences in *PCNA* expression within the rostral HF did not co-vary with serum CORT ($F_{1,24.1}$ = 0.14, p = 0.708), blood glucose ($F_{1,38.8}$ = 0.24, p = 0.626), H:L ratio ($F_{1,43.9}$ = 2.91, p = 0.095), mealworm consumption ($F_{1,47.7}$ = 0.04, p = 0.836), or relative spleen mass ($F_{1,49.9}$ = 4.68, p = 0.035). There were no interactions with hemisphere.

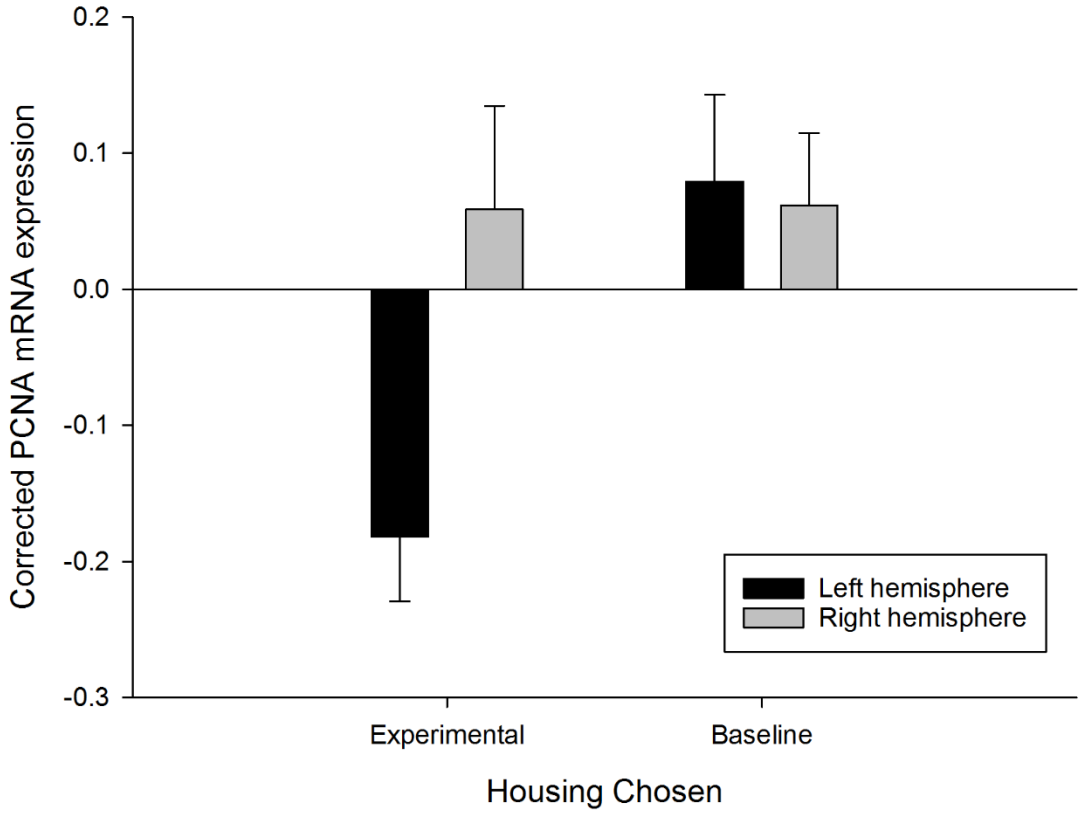

Figure 2.5. Mean expression of mRNA for proliferating cell nuclear antigen (PCNA) in the rostral HF of hens that chose either their experimental housing or the baseline/intermediate housing in two-way preference tests. P:Experimental; P-housed hens that chose P conditions (n=11), P:Baseline; P-housed hens that chose baseline conditions (n=4), NP:Baseline; NP-housed hens that chose baseline conditions (n=11), NP:Experimental; housed hens that chose NP conditions (n=4). Error bars represent +1 standard error. Gene expression values are residual after correcting for individual (hen), LBR expression and pen, in LMMs.

For cDNA reverse transcribed in a single batch of all HF samples taken from the left hemisphere, gene expression was analysed in four separate models. According to the Bonferroni correction, the threshold of α ≤ 0.0125 was used to detect significant effects. In samples from the left hemisphere alone, *DCX* expression did not differ between the rostral and caudal subregions ($F_{1,27.3}$ = 0.82, p = 0.374). There was not a main effect of housing ($F_{1,76.5}$ = 0.03, p = 0.874), nor of choice ($F_{1,86.2}$ = 1.06, p = 0.306). Perhaps due to the smaller sample size, no interactions reached significance. As a proxy for the proportion of immature neurons relative

to mature neurons present in the HF, *DCX* mRNA was also explored in relation to *NeuN* mRNA as a covariate. With this alternative housekeeping gene, *DCX* expression still did not differ with subregion ($F_{1,26.0} = 1.07$, $p = 0.311$), housing condition ($F_{1,25.3} < 0.001$, $p = 0.991$) or choice ($F_{1,26.0} = 1.17$, $p = 0.290$), and there were no significant interactions.

In samples from the left hemisphere, the *MR*/*GR* expression ratio was not significantly different between samples from the rostral and caudal HF ($F_{1,20.6} = 5.99$, $p = 0.024$), nor between hens housed in P and NP conditions ($F_{1,21.0} = 0.01$, $p = 0.919$) or that made the majority versus minority choice ($F_{1,21.8} = 1.77$, $p = 0.198$). There were no significant interactions.

Expression of mRNA for *DCLK1* (also expressed in immature neurons) was additionally quantified in samples from the left hemisphere. *DCLK1* expression also did not differ between subregions ($F_{1,23.2} = 0.69$, $p = 0.413$) or with housing condition ($F_{1,25.2} = 0.42$, $p = 0.521$) or choice ($F_{1,25.9} = 0.11$, $p = 0.739$). No interactions reached the significance threshold for either gene. Serum CORT did not co-vary with *DCLK1* expression in the left HF ($F_{1,26.0} = 1.23$, $p = 0.278$), and the same was true for blood glucose ($F_{1,25.8} = 0.005$, $p = 0.944$), H:L ratio ($F_{1,24.6} = 0.09$, $p = 0.768$), mealworm consumption ($F_{1,25.1} = 1.10$, $p = 0.305$) or relative spleen mass ($F_{1,24.8} = 6.31$, $p = 0.019$). No covariate interacted with HF subregion.

2.5 Discussion

2.5.1 Effect of housing conditions

Results of the preference tests conducted at the end of the study indicate that the "Preferred" and "Non-Preferred" housing conditions were successfully designed, as the former were more often chosen over the intermediate housing conditions. However, none of the physiological (serum CORT, blood glucose, H:L ratio, relative spleen mass) or behavioural (mealworm consumption) parameters measured differed with housing condition. There are two possible explanations. The first is that the difference between the experimental housing alternatives was not dramatic enough to influence the overall experience of hens. In this case, the P housing was not sufficiently enriching to produce positive affect, and/or the NP housing was not barren or aversive enough to cause chronic stress. Accessing nest boxes and perches is known to be a high behavioural priority for hens (Cooper and Appleby, 1996a; Olsson and Keeling, 2002), but some form of nest box and perch was provided in all environments, and it may be that the poorer alternatives were sufficient to prevent stress. Though the NP environment did not provide any dust bath, whereas the P housing contained a dust bath of preferred substrates (van Liere and Siard, 1991), some studies suggest that dust bathing is afforded a relatively low behavioural priority by hens (Bubier, 1996b; Weeks and Nicol, 2006). It is therefore possible

that the welfare of birds was not negatively impacted by the more basic (NP) housing conditions.

However, the housing conditions were evidently salient enough to influence the choice made by the majority of hens (in favour of the P housing, & avoiding the NP housing). The second possibility is therefore that the measures taken were not sensitive enough to detect a potentially subtle effect of housing on the hens, which was reflected only by their autonomous decision making. As the physiological variables assessed generally reflect chronic stress, it might be that neither housing environment was stressful, but that positive affective experience associated with the P housing could not be detected. A previous study with a similar design (Nicol *et al.*, 2009) compared the preferences of hens for *1)* a pen providing foraging materials (litter & peat), nest boxes and perches, *2)* a barren pen with wood shavings litter, and *3)* a barren pen with a wire floor. Overall, hens significantly preferred the former environment to the latter, but did not significantly choose it over the pen containing only shavings, and exhibited no difference in preference for the barren pens including or lacking litter. As the differences between the inanimate environments compared in the present study were less dramatic, it is possible that the more discerning preferences observed can be attributed to the concurrent experience of the positive reinforcers and punishers. These rewards and stressors may have led to hens forming positive and negative associations during their time in the P or NP housing respectively, which informed decisions in the subsequent preference tests.

While AHN appears to be a sensitive proxy for long-term experiences with both positive and negative valence in rodents (section 1.4.3 & 1.4.4), and chronic negative stress suppresses AHN in the chicken HF (Robertson *et al.*, 2017; Gualtieri *et al.*, 2019), expression of none of the quantified mRNA transcripts differentiated between hens from the P and NP housing conditions in a simple manner. Due to the similar lack of housing effects on the other (physiological) measures recorded, it is not possible to determine whether these null effects are due to *i)* a lack of differential impact of the experimental housing conditions on overall experience, *ii)* transcription comprising a poor proxy for the rate of neurogenesis, or *iii)* a lack of sensitivity of AHN in chickens to conditions that did (perhaps positively) influence cumulative experience, in a manner that could not be detected. While future work must seek to address these questions, some of the measures taken were instead associated with the choices made by hens in the preference tests, suggesting that individual differences may have exceeded differences between the assigned groups.

2.5.2 Relationship with preference

Interestingly, while serum CORT was not influenced by housing condition, this measure of HPA-axis activity was influenced by choice in the preference tests. For both housing conditions, hens that made the minority (unexpected) choice were characterised by higher basal CORT than hens that made the majority choice. Half of these hens selected the intermediate housing over their P experimental housing, while the other half selected their NP experimental housing over the (objectively better) intermediate conditions. Though individual hens differ in their preferences for particular types of inanimate housing environment (Nicol *et al.*, 2009), the fact that the two subsets of minority hens made choices in favour of different conditions makes them unlikely to represent a single group with common traits. When samples from the caudal HF were analysed in a single qPCR batch, expression of *DCX* mRNA was also associated with this choice. Hens making the minority choice from both housing systems had lower *DCX* expression. This pattern mirrors the difference in CORT levels, and is consistent with the causal mechanism whereby stress-induced elevations in CORT suppress AHN (Lehmann *et al.*, 2013). In the measurement of mRNA over all HF samples, P hens making the minority choice had lower average *DCX* expression in both HF subregions than P:majority hens, while NP hens making the minority choice had lower average *DCX* expression in the caudal HF but higher expression in the rostral subregion. Perhaps due to variability between the two processing batches, few of these pairwise comparisons were significant. While there may be issues regarding methodological reliability, discussed further below, the caudal avian HF is hypothesised to be preferentially responsive to stress (Smulders, 2017; Gualtieri *et al.*, 2019), and the associated results for *DCX* mRNA expression shall therefore be considered.

It is possible that both higher basal CORT and lower AHN are purely intrinsic characteristics of two minority subgroups of hens, with a stable relationship with their (atypical) preferences and decision-making. On the other hand, these observations could also be explained by an interaction of intrinsic differences, and/or differences in non-shared experiences, with housing conditions. Lower basal CORT and higher rates of AHN are associated with greater resilience to stress (Anacker and Hen, 2017), as adult-born neurons inhibit mature neurons responsive to anxiogenic conditions (Anacker *et al.*, 2018) and may prevent generalisation of negative associations (Anacker and Hen, 2017). In line with suppressing AHN, there is evidence that the chronic experience of stress may negatively impact future resilience (Peña *et al.*, 2019). P hens that chose the baseline conditions may have (for individual reasons) experienced stress in their housing environment, leading them to form a negative association with those conditions, which in turn motivated them to choose an alternative. Conversely, the NP environment was designed

to produce a shared negative experience, which may have led to negative associations, and a consequent preference for the baseline housing, in the majority of hens. However, a minority of less resilient hens (due to intrinsic factors or relatively more stressful experiences), exhibiting higher basal CORT and lower *DCX*, may have responded to the additional strain by effectively "giving up", and accepting the familiar (NP) housing. Repeated stress leads to learned helplessness in susceptible rodents, as a subset of rats have been observed to no longer attempt to escape a previously unavoidable foot-shock once this becomes possible (Malberg and Duman, 2003). Chronic stress also causes mice to spend more time immobile during the forced swim test (Lehmann *et al.*, 2013), and there is individual variation in this expression of behavioural despair (Estanislau *et al.*, 2011). In rats, learned helplessness was previously associated with impaired cell proliferation and survival specifically in the most temporal third of the hippocampus (Ho and Wang, 2010), while NP:minority hens also had lower *DCX* expression in the temporal (caudal) HF than in the septal (rostral) subregion, which may support this explanation. Experiences of stress during adulthood may also promote subsequent avoidance of novel stimuli or environments, with inescapable shocks causing neophobia (of a novel context or odour) in rats (Minor, 1990). Fear associated with the less familiar intermediate housing conditions may also have motivated the choices of the NP-housed hens that showed an apparent preference for these conditions.

An interpretation which differentiates between hens that made the minority choice from P and NP housing is also consistent with quantified *MR*/*GR* mRNA expression ratios. The MR/GR ratio is known to be reduced in the HF of mammals (Liberzon *et al.*, 1999; Zhe *et al.*, 2008) and birds (Dickens *et al.*, 2009; Banerjee *et al.*, 2012) by prolonged stress. In the present study, the hippocampal *MR*/*GR* ratio was reduced specifically in those NP housed hens that made the minority choice, with this effect localised to the rostral HF subregion. Alterations to the MR/GR ratio may reflect individual differences in susceptibility to the pathophysiological effects of chronic stress. In a mouse model of PTSD, hippocampal MR/GR ratios were decreased in individuals displaying stress-induced alterations in negative feedback to the HPA-axis, but quickly normalised in those individuals that maintained normal glucocorticoid feedback (Liberzon *et al.*, 1999). This may support the interpretation that the NP-housed hens that did not choose to avoid these housing conditions were in fact the most stressed. As well as potentially attenuating resilience and leading to a learned helplessness-type response (as in Malberg and Duman, 2003), stress may negatively impact the cognitive processes required for rational decision making, such as recognition memory (Kim *et al.*, 2013).

Expression of mRNA for proliferative marker *PCNA* in the rostral HF exhibited a different pattern, and was positively associated with preference for the baseline environment. Expression of *PCNA* was thus higher both in NP hens that made the majority choice, and in P hens that made the minority choice. These results are consistent with *DCX* expression for the NP hens, but not for P hens. While it is possible that a preference for particular features of the baseline environment was associated with *PCNA* expression, the fairly subtle nature of the difference in conditions (especially compared to the P housing) makes this seem unlikely. It may instead be the case that hens with higher levels of HF proliferation tend to choose the environment that they have not recently lived in. The previous study of housing preferences in hens found a strong negative effect of the environment that the birds were housed in prior to testing on their probability of choosing that option (Nicol *et al.*, 2009). This preference for novelty might comprise an intrinsic factor with influence on individual-specific preferences. Expression of *PCNA* mRNA in the hippocampal homologue has previously been associated with reactive versus proactive personality types, or behavioural strategies, in Atlantic salmon (*Salmo salar*) (Vindas *et al.*, 2017). Generally, reactive individuals are more sensitive to external cues and display greater behavioural flexibility, while proactive individuals tend to respond in a rigid, routine-like manner (Benus *et al.*, 1991; Coppens *et al.*, 2010). This may have led more proactive hens to choose the familiar environment to which they were habituated, while reactive hens sought to gather information on alternative options and thus selected the conditions that they had not been living in. The reason that *PCNA* expression differed with choice for baseline or experimental housing only within the left hemisphere is unclear, though reactive and proactive coping behaviours have been suggested to be preferentially associated with activation or dominance of the right and left hemisphere respectively (Tops *et al.*, 2017). This dissociation requires further exploration in the context of AHN.

2.5.3 Methodological consistency & conclusions

While repeat measurements of *DCX* mRNA transcripts in the same HF tissue subsamples were significantly correlated, the relationship was notably stronger when cDNA from the same reverse transcription batch was used for the qPCR assays, compared to when the RNA employed was reverse transcribed separately. This suggests that technical variability introduced at the stage of reverse transcription might obscure certain relationships. While measurement of control gene *LBR*, expressed by all nucleated cells (Olins *et al.*, 2010), should account for variable efficacy of cDNA amplification and reverse transcription processes between samples, this technical control measure does not appear to have been entirely successful in the latter instance. As such, the absence of relationships between HF gene expression and preference test

choices in the single batch of samples from the left hemisphere could be attributable either to the lower statistical power of the smaller sample size, or to technical variability between the two reverse transcriptions. This raises questions as to the reliability of the associations between HF gene expression and housing choice in the larger set of samples. However, differences observed were not especially dramatic, and the primary aim of the study was instead to identify biomarkers reflecting the long-term experience of housing conditions designed to be relatively positive or negative for the residing hens. As neither the existing physiological and behavioural measures of stress taken, nor gene expression in the HF, discriminated between the two experimental groups, it is difficult to assess the validity of transcript abundance in HF tissue as a proxy for experience in the present study. At the level of the individual, hippocampal gene expression was also not correlated with variation in any other measure. However, given the wide range of factors with known influences on AHN, it is unclear whether a linear relationship with any single factor measured would have been expected.

Overall, while hens demonstrated a greater preference for the conditions designed to be preferred than for the conditions designed to be non-preferred, neither the physiological and behavioural measures taken nor expression of target mRNA transcripts in the HF distinguished between birds from the two housing environments. Individual differences in choice, however, related to serum CORT and exhibited some associations with hippocampal expression of *DCX*, *MR*/*GR* and *PCNA*. While all of these measures may lack sensitivity, particularly in their capacity to detect positive experiences, the findings suggest that the provision of different forms of individual resources within housing systems may not have dramatic implications for the overall experience of commercial hens. Individual differences, reflected by preference, may be associated with variation in physiology relating to the stress response, and in indices of hippocampal plasticity.

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Chapter 3. Neurogenesis in the Adult Chicken Hippocampus Correlates with Individual Differences in Time Spent in Outdoor Areas and Tonic Immobility

3.1 Abstract

Access to outdoor areas is provided as a means of enhancing welfare in commercial systems for laying hens, but proportions of time spent on the range by individual birds vary substantially. Adult hippocampal neurogenesis in mammals and birds is upregulated by positive experiences including environmental enrichment and exercise, while basal levels of proliferation have been associated with individual differences in reactive versus proactive coping style. We thus sought to explore whether individual differences in use of outdoor areas and in tonic immobility (TI) responses (indicative of fearfulness) were associated with hippocampal cell proliferation and neuronal differentiation. Radio frequency identification technology was used to track the ranging behaviour of 440 individual focal hens within a commercially relevant system over a 72-day period, after which tonic immobility durations were measured. Following hippocampal tissue collection from 58 focal hens, proliferation and neuronal differentiation were measured through quantitative PCR for proliferating cell nuclear antigen (*PCNA*) and doublecortin (*DCX*) mRNA respectively. Variation in ranging was not related to TI responses. Greater time spent in outdoor areas (the grassy range and stone yard) was associated with higher *PCNA* expression in the rostral subregion, while individual differences in TI duration positively correlated with proliferation over the whole HF. Basal proliferation in the chicken HF may thus be an indicator of reactivity, while levels in the rostral region may be stimulated by ranging experience. *DCX* expression in the caudal HF negatively co-varied with time on the range, but was not associated with TI duration. This suggests that ranging outside may also be associated with stress. Within laying hen flocks, individual differences in hippocampal plasticity thus relate independently to coping style and use of external areas.

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3.2 Introduction

Within commercial flocks of laying hens, variation between individuals may be associated with differing experience and overall welfare. For example, many systems provide access to outdoor areas as a form of enrichment, which expands freedom of movement, behavioural repertoire, exploration and foraging opportunities for hens, beyond those already afforded by the barns (Knierim, 2006; Chielo *et al.*, 2016). Outdoor ranges may also offer an environment of greater unpredictability than the barn interior, where conditions are tightly controlled (Campbell *et al.*, 2018). However, there is substantial variation in the extent to which individual hens use these external areas. Radio frequency identification (RFID) tracking within flocks consistently highlights distinct subgroups, wherein a proportion of hens access the range daily, while others seldom or never venture outside (Richards *et al.*, 2011; Gebhardt-Henrich *et al.*, 2014; Campbell *et al.*, 2017; Larsen *et al.*, 2017).

An association between differential ranging experience and the affective state of hens is supported by behavioural tests of fearfulness. Indoor-preferring birds exhibit longer durations of TI (Grigor *et al.*, 1995; Hartcher *et al.*, 2016), are more vigilant and slower to feed following playback of an alarm call (Campbell *et al.*, 2019), produce fewer vocalisations during manual restraint, and move less in open field tests (Campbell et al., 2016). However, it is not clear whether these responses are moderated by ranging experience, or if common intrinsic factors drive both fear behaviour and ranging propensity. Additionally, use of the range has been positively correlated with the plasma CORT response to handling and behavioural testing (Larsen *et al.*, 2018), and aspects of ranging such as lack of cover (Singh and Cowieson, 2013), risk of predation (Nicol *et al.*, 2011), exposure to the elements and to a greater burden of pathogens (Lay Jr. *et al.*, 2011) may be stressful. Recent research also indicates that frequently ranging hens are less inclined to be near to their conspecifics (Ferreira *et al.*, 2020a). This could reflect independence/boldness, or alternatively a motivation to utilise the low stocking density of the range (Chielo *et al.*, 2016) to escape social conflict. Several other behavioural, physiological and health-related indicators of hen welfare have been reported to demonstrate no notable associations with relative outdoor range usage (Larsen *et al.*, 2018). As such, the implications of individual ranging experience for cumulative stress are currently unclear.

Hens form stable routines in terms of daily activity (Larsen *et al.*, 2017; Rufener *et al.*, 2018) and individual ranging behaviour is consistent over time (Marino, 2017; Garnham and Løvlie, 2018; Ferreira *et al.*, 2019). Levels of adult hippocampal neurogenesis (AHN) are modulated by long-term experience, but whether they are associated with ranging activity has yet to be

determined. In the mammalian brain, directional changes in rates of hippocampal proliferation and survival distinguish negative stress from positive eustress. Enriching experiences including environmental complexity (Bruel-Jungerman *et al.*, 2005), exercise (Van Praag *et al.*, 1999) and learning (Gould *et al.*, 1999) upregulate AHN, often preferentially in the septal DG (Tanti *et al.*, 2012; Nishijima *et al.*, 2013; Ramirez-Rodriguez *et al.*, 2014; Gualtieri *et al.*, 2017). Conversely, chronic negative stress suppresses AHN and may have particular influence in the temporal subregion (Jayatissa *et al.*, 2006; Hawley and Leasure, 2012; Hawley *et al.*, 2012; Tanti *et al.*, 2012; Tanti *et al.*, 2013). Increases in AHN driven by environmental enrichment and exercise are typically accompanied by a reduction in anxiety- and depressive-like behaviours in mice (Duman *et al.*, 2008; Schloesser *et al.*, 2010; Hendershott *et al.*, 2016), while these are increased by prolonged stress (Elizalde *et al.*, 2010; Lehmann *et al.*, 2013). In birds, AHN levels are similarly stimulated by environmental enrichment and complexity (LaDage *et al.*, 2010a; Melleu *et al.*, 2016) and downregulated by chronic stress (Barnea and Pravosudov, 2011). The rostral avian HF, posited to be homologous to the septal rodent subregion (Smulders, 2017), may be more involved in spatial cognition and memory (Smulders and DeVoogd, 2000; Smulders *et al.*, 2000), while AHN in the caudal laying hen HF appears especially sensitive to stress (Gualtieri *et al.*, 2019). The relationships between individual levels of ranging, fear-related behaviour and AHN within the respective HF subregions may therefore reflect whether ranging is generally an enriching or stressful experience for hens.

On the other hand, variation in ranging propensity and fear behaviour may relate to aspects of personality, defined as consistent inter-individual differences in behaviour (Roche *et al.*, 2016). A well-characterised dimension of animal personality is the tendency to adopt an active (or proactive) versus passive (or reactive) behavioural strategy when challenged (Erhard and Mendl, 1999), also referred to as a coping style (Benus *et al.*, 1991). Reactive/passive individuals are predisposed towards displaying a freezing-type fear response as opposed an active fight or flight response (Erhard and Mendl, 1997), and are thus more easily induced into immobility and remain in this state longer (Erhard and Mendl, 1999; Edelaar *et al.*, 2012). Consequently, individual differences in reactivity for hens may be reflected in their durations of TI: a catatonic-like freezing response induced by brief physical restraint in an upturned position (Broom, 1989). Consistent with a personality trait, variation in duration of the TI response is heritable (Gallup *et al.*, 1976; Nakayama *et al.*, 2010). In line with freezing less, proactive individuals are more prone to exploration (Benus *et al.*, 1990; Hall *et al.*, 2015).

Behavioural strategy also relates to individual differences in speed versus accuracy during learning, with proactive individuals acquiring simple novel tasks more quickly (Sih and Del

Giudice, 2012; Zidar *et al.*, 2018). For example, black-capped chickadees (*Poecile atricapillus*) that readily enter a novel environment are faster to learn an acoustic discrimination task (Guillette *et al.*, 2009). In hens, proactive behaviour has been shown to predict predisposition to use the outdoor range. When tested before any range access, pullets from an enriched rearing environment that were quickest to reach T-maze success (presumably pro-active) also proceeded to visit the range most frequently over the four successive weeks (Campbell *et al.*, 2018). However, while proactive individuals tend to maintain rigid, routine-like behaviour, reactive individuals are more sensitive to changes in the environment/task requirements and display enhanced behavioural flexibility (Benus *et al.*, 1991; Coppens *et al.*, 2010; Höglund *et al.*, 2017). Rats selectively bred for their ability to learn new configurations in a maze task were more susceptible to TI and slower to explore a novel environment than those bred for low maze performance (McGraw, 1973). In birds, more explorative red junglefowl hens (Zidar *et al.*, 2018) and black-capped chickadees (Guillette *et al.*, 2010) were slower at reversal learning, while behavioural flexibility was positively correlated with fearfulness in junglefowl chicks (Zidar *et al.*, 2019). Moreover, low ranging broiler chickens improved in accuracy of spatial discrimination between trials of a memory task occurring on the same day, whereas higher rangers behaved inflexibly and did not alter their performance on the second trial (Ferreira *et al.*, 2019). Compared to higher rangers, chickens that ranged less were also better at inhibiting their behaviour by detouring to the sides of a transparent cylinder to access a food reward, rather than pecking the cylinder walls (Ferreira *et al.*, 2020b).

Interestingly, behavioural strategy is reflected in levels of proliferation in the hippocampus. Quantitative PCR indicated that Atlantic salmon (*Salmo salar*) characterised as reactive had a higher basal expression of PCNA mRNA in the hippocampal homologue than their proactive conspecifics (Vindas *et al.*, 2017). Furthermore, cell proliferation in rats that predominantly responded to a novel environment by freezing was twice that observed in proactive rats, while proliferating cell numbers positively correlated with durations of freezing on an individual level (Lemaire *et al.*, 2008). The rigid and inflexible behaviour displayed by proactive individuals has recently been linked to limitations in their neural plasticity (Øverli and Sørensen, 2016), and a causal role of newborn cells in flexible spatial behaviour has been demonstrated through experimental suppression of AHN. Mice with experimentally ablated AHN are impaired in learning a changed (reversed) goal location in a water maze (Garthe *et al.*, 2009), and in avoiding a rotating shock zone when this is added to a stationary zone learnt first (Burghardt *et al.*, 2012). As such, an association between proliferation in the hippocampus and behavioural strategy may relate to the requirement of plasticity for flexible spatial behaviour.

In the present study, RFID tags were used to track individual ranging behaviour in terms of the proportional time that hens spent in four distinct areas: 1) the barn, 2) an adjoining covered winter-garden, 3) an adjacent uncovered stone yard, and 4) a large, grassy range. This set-up may also facilitate separation of the implications that various aspects of the environment have for hen behaviour and AHN. For example, the winter-garden provides fresh air but cover from rain, both the stone yard and range are exposed to the elements and to predators, and the range alone provides grass. Ranging a greater distance from the barn has been positively associated with welfare parameters in broiler chickens (Taylor *et al.*, 2020). Following longitudinal recording of ranging, TI durations were measured and hippocampal expression of *PCNA* and *DCX* mRNA was quantified. The first possibility is that AHN is modulated by ranging experience, with consequences for affective state. Given that ranging outdoors is likely associated with additional environmental complexity and exercise, this behaviour might be predicted to upregulate AHN while reducing anxiety. If so, individual differences in AHN should correlate positively with time on the range (and possibly the stone yard) but negatively with durations of TI. However, if ranging is stressful, time outside/on the range would correlate negatively with AHN and positively with TI. It is also possible that ranging is simultaneously enriching and stressful, and based upon putative subregional specialisation in the HF, these influences would be predicted to preferentially relate to AHN in the rostral and caudal subregions respectively. The second possibility is that individual differences in ranging stem from personality type, whereby low-ranging (less explorative) hens are reactive and thus also more fearful and behaviourally flexible. As such, we would predict pro-active hens with shorter TI times to be more likely to explore the range, and AHN throughout the entire HF should co-vary negatively with ranging and positively with TI.

3.3 Methods

The following experiment was conducted at the Centre for Proper Housing: Poultry and Rabbits (ZTHZ), University of Bern, by a team led by Dr. Michael Toscano. This group was responsible for the experimental design, husbandry and the collection of tonic immobility and ranging data. Hippocampal tissue collection, processing of samples for molecular biology, quantification of mRNA and all reported statistical analyses were conducted as part of the submitted doctoral research project.

3.3.1 Ethical statement

Experimental use of the animals was approved by the Bern Kantonal Authority (BE-46/16) and the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549), and

procedures complied with Swiss regulations regarding their treatment. Standard commercial protocols were followed, including ad libitum access to food and water.

3.3.2 Animals & facilities

Following on-site rearing (detailed in Guerrero-Bosagna *et al*., 2020). 17-week-old Brown Nick (H&N International) laying hens were transferred to a commercial laying hen house at the Aviforum (Zollikofen, Switzerland). Only one of the barn's two halves was used for the present study, wherein pens were equipped with a system that allowed the tracking of individual animals. The four study pens (each 12.9 m^2) contained a Rihs Bolegg II commercial aviary system (Krieger AG, Ruswil, Switzerland) with a stocking density of 9.33 hens/m^2. The aviary structure and group nests lined one wall, and the floor of the barn was covered with 10 cm of wood shavings. The aviary was 2.40 m high and consisted of three tiers, with integrated equipment comprising: a manure belt, feeding chain, and nipple drinkers within the lowest tier; a manure belt within the middle tier; and a feeding chain and nipple drinkers within the highest tier. Plastic mushroom-shaped perches were provided on the lowest and highest tiers and plastic platforms to move between tiers were provided along both aviary sides (30 cm in width and at 70 cm height from the floor). Nest entries were square plastic grids (size 2.5 × 5 cm). External to the barn were three separate areas: a winter-garden, stone yard and grassy range, each linked at a single location (pophole or gate) to facilitate sequential movement of birds when open, but closed to limit access as required by the management protocol. Fencing between pens maintained divided populations within all (internal and external) areas. Adjacent to the barn interior, birds had access to the winter-garden (~17.55 m^2 per pen), which was entirely covered by a solid roof and surrounded by wire mesh on the sides and in between pens. The floor of the winter-garden was lined with a thin layer of wood shavings of the same type provided within the barn, and the area was equipped with nipple drinkers and perches. A manually operated pophole separated the winter-garden from an uncovered yard area (~88 m^2 per pen) which was lined with small stones and enclosed by a fence. Beyond a gate in the stone yard was the "free-range": an open, grassy pasture with an average size of 288 m^2 per pen. The grass was routinely mowed, and access was restricted during periods of dry weather to ensure it was maintained. Upon introduction to the barn, 355 hens were placed into each of the four pens, and 110 randomly selected birds per pen were fitted with an RFID transponder (Hitag S 2048 bits, 125 kHz) attached to an adjustable leg band (IDs, Roxan, Scotland). Artificial light was provided in the barn from 0200 to 1700 h, with transitional phases of five minutes beginning at 0200 h and 15 minutes beginning at 1645 h. Natural daylight was provided from 0800 to 1630 h through windows controlled by curtains. To allow hens to acclimatize to the barn interior, they were

kept inside for the first week. Subsequent access to the winter-garden, stone yard and range was first provided one, two and four weeks after population respectively. For the subsequent five-month period (June 7 to October 16, 2016), birds were permitted weather-dependent voluntary daily access to the external areas. Antennae were positioned on either side of the transition points (popholes/gates) connecting two areas and RFID transponders recorded the date and time of each zone-transition made. Records permitted calculation of the time spent in each area (as in Gebhardt-Henrich *et al.*, 2014), but not distances travelled within them. At the conclusion of the daily period for which birds were provided outside access, those in other areas were encouraged back into the barn interior. At 42 weeks of age, a roughly equal number of tagged birds from each pen were haphazardly selected for sampling of hippocampal tissues (total n=58). Loss of samples from five birds during molecular biology processing resulted in a final sample size of 53 birds.

3.3.3 Ranging quantification

Daily observations started at the time the range was opened and stopped when the range was closed. Based upon weather conditions, daily opening times varied between 7:50 and 13:50 and closing times ranged from 14:20-16:55, though hens were typically allowed to range until 16:30 each day. On days that behavioural testing, management protocols (vaccinations) or poor weather required restricted access to outdoor areas, access was intentionally restricted equally across all pens. The percentage of time spent in each area by each hen was calculated based on the remaining observation period of 72 days, wherein the range was accessible for an average of 6 h 58 min per day. There were some instances of loss of antenna signal coverage, generally caused by birds moving too quickly for detection. This meant that not all transitions were recorded. However, data checks confirmed accurate recording of each animal's movement and location patterns (e.g. sequential progression through areas rather than “jumps” to more distal ones). On average, the location of a bird was recorded for 83% of the time the range was open (mean coverage rate, IQR 69-96%). Proportions of available time spent in the different areas by each bird were calculated based only upon times wherein their individual zonal locations were transmitted, leaving an average of 5 h 47 min tracked per day. As missed recordings were distributed evenly over all areas, while the actual times that birds spent on the range may have been higher than observed, the proportional times analysed should not have been affected. Detailed data regarding movement of hens between the barn and external areas is reported elsewhere (Guerrero-Bosagna *et al.*, 2020).

3.3.4 Tonic immobility

As part of related experimental evaluations (reported elsewhere: Vogeli, 2017), collection of final measurements spanned a four-day period in which all hens were prevented from leaving the barn. On each day, hens from a single pen were transported from their home pen to another barn on-site for the measurement of TI, which occurred shortly before tissue collection. To induce immobility, hens were placed on their backs on a holding frame, with a light pressure applied to the breast. After pressure was released, the latency until the hen righted itself was timed using a stopwatch. The same observer (SGH) conducted all TI tests. If immobility was not successfully induced (i.e. the bird started to move within 3 seconds of removal of pressure), the procedure was repeated up to 3 times. Where immobility was not induced after the final attempt, the hen received a latency of 0 seconds. If a hen remained immobile for 300 seconds, they were ascribed this value as the maximum latency and the test was terminated.

3.3.5 Tissue collection

Shortly after TI measurement, animals (n=58) were killed via intravenous injection with pentobarbital (Esconarkon, 0.3 ml/hen due to similar body weights). Immediately thereafter, brains were removed from the skull, placed into 0.1 M phosphate-buffered saline in a Petri dish and divided along the longitudinal fissure with a scalpel. From each hemisphere, the HF was dissected and divided midway across the rostrocaudal axis to produce two subsamples (rostral & caudal) containing equal amounts of tissue. The four HF samples collected from each hen were processed separately. Isolated HF regions were placed in sample tubes containing 1.5 ml of RNAlater® Stabilization Solution (Thermo Fisher Scientific, UK) and refrigerated for 24 hours before storage at -30°C.

3.3.6 RNA isolation & reverse transcription

RNA was extracted according to the methods described previously (section 2.3.6), using TriSure reagent (Bioline, London, UK) and Lysing Matrix D tubes in a FastPrep Instrument (MP Biomedicals, Cambridge, UK). Purification of the RNA product combined with DNAse treatment was conducted with the Zymo Direct-zol™ RNA MiniPrep Kit (Cambridge Bioscience, Cambridge, UK), according to manufacturer's instructions. 2µg RNA was reverse transcribed using the Tetro™ cDNA Synthesis Kit (Bioline, London, UK) for use in a quantitative real-time polymerase chain reaction (qPCR).

3.3.7 Quantitative PCR

As before, the chicken lamin B receptor (LBR) gene was used as a control gene for normalisation (Dunn *et al.*, 2013). Gene specific primers for *PCNA*, *DCX* and *LBR* were previously designed using the NCBI primer-BLAST tool, with sequences displayed in Table 2.1. Standards were produced by gel purification of PCR products (as in section 2.3.7) using a MinElute gel extraction kit (Qiagen Ltd, Crawley, UK) and their concentration was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). Serial dilutions of standards were produced to create standard curves for qPCR quantification. qPCR reactions were run on a Bio-Rad model machine (Bio-Rad, California, USA). Reactions (20 µl) contained 5 µl of cDNA template together with 10 µl SYBR green master mix (No-ROX kit, Bioline, London, UK) and gene specific primers (400 nM). The manufacturer's instructions were followed for 3-step thermal cycling conditions. Samples were run in singlicate over three batches on a 96-well plate, accompanied by a standard curve run in duplicate. No-template controls were also included. A melting curve analysis was performed to confirm specificity of reactions and efficiency values for the primers used ranged between 99.7% and 108.9%. Assays were analysed using CFX-Manager software (Bio-Rad, California, USA).

3.3.8 Statistical analysis

Analyses were conducted in IBM SPSS Statistics (v24). Linear mixed models (LMMs) were conducted to explore how the proportional times spent in each of the four areas (barn, winter-garden, stone yard and range) by hens were related, while accounting for experimental pen as a random factor. Times in the intermediate areas (winter-garden and stone yard) were included separately as covariates while times in the extreme areas (barn and range) were dependent variables. Whether time in the winter-garden co-varied with time in the stone yard was also explored. Separate univariate ANOVAs were employed to determine whether TI durations and time spent in each area differed between experimental pens. To explore whether ranging was related to TI durations, LMMs were conducted with TI duration as the dependent variable, pen as a random factor, number of attempts to induce TI as a fixed factor, and proportional time in each of the areas as covariates (over four individual models). Measured molar quantities of *PCNA*, *DCX* and *LBR* mRNA were log(10)-transformed and, as the quantity of samples necessitated multiple qPCR runs, normalised using the Standard Score (Z_i) within assays. Separate LMMs with unstructured covariance were conducted for *PCNA* and *DCX*, each with HF subregion (rostral/caudal) and sample (one per hemisphere) as repeated fixed factors, pen as a random factor and *LBR* expression in the same sample as a covariate. In individual models, percentage of time spent in each area was included as a covariate, as well as in their interaction

with HF subregion. Two models explored whether TI was related to expression of each gene and included TI duration as a covariate, TI attempts as a fixed factor, and both variables in an interaction term with HF subregion. Where both TI duration and time in an area co-varied significantly with expression of the same gene, they were also included together in a single LMM to verify their explanation of independent proportions of the variance. The corrected gene expression values plotted in Figures 3.2 & 3.3 comprise residual *PCNA* and *DCX* after accounting for *LBR* expression, HF subregion, sample and pen in LMMs, as described above.

3.4 Results

3.4.1 Behaviour

On average, the 53 focal hens spent the majority of available tracked time either in the barn or in the winter-garden (see Table 3.1). Proportional time in the winter-garden was therefore negatively correlated with time spent in the barn ($F_{1,50.9} = 199.7$, $p < 0.001$, B = -1.212, SEM = 0.086). One hen remained exclusively within the barn and never entered the winter-garden. Time spent in the winter-garden was positively correlated with time spent in the stone yard ($F_{1,50.5} = 11.06$, $p = 0.002$, B = 0.161, SEM = 0.049), but was not related to time on the grassy range ($F_{1,50.5} = 1.04$, $p = 0.313$, B = 0.052, SEM = 0.051). Five hens never ventured outside (i.e. to the stone yard), while an additional three spent time in the stone yard but did not enter the grassy range. This meant that 45 hens (~85%) thus used all areas provided to some extent. Time spent in the stone yard was positively correlated with time on the range ($F_{1,50.5} = 18.12$, $p < 0.001$, B = 0.494, SEM = 0.116).

The mean number of daily transitions that hens made between areas correlated negatively with time in the barn ($F_{1,49.3} = 118.75$, $p < 0.001$, B = -0.9753, SEM = 0.089) and positively with time in each of the three other areas (winter-garden: $F_{1,49.6} = 49.95$, $p < 0.001$, B = 0.5895, SEM = 0.087; stone yard: $F_{1,49.3} = 46.84$, $p < 0.001$, B = 0.2236, SEM = 0.033; range: $F_{1,49.5} = 20.72$, $p < 0.001$, B = 0.1670, SEM = 0.037).

Table 3.1. Descriptive statistics for proportions of available time (%) spent in each of the four areas of the housing system by focal hens (n=53) over the tracked period. "Hens visited" refers to the number of birds that spent at least some time in each area, though all hens were included in the calculation of statistics.

	Hens Visited	Mean	Standard deviation	Median	IQR	Range
Barn	53	51.31	26.61	41.00	51.64	11.81 – 100.00
Winter-garden	52	34.54	19.61	40.47	35.22	0.00 – 66.07
Stone Yard	48	8.10	7.70	7.16	11.37	0.00 – 33.72
Range	45	6.05	7.30	2.28	7.90	0.00 – 28.28

Behaviour was compared between the four experimental pens. There was no difference in durations of TI between pens ($F_{3,47} = 1.10$, $p = 0.358$), but the number of attempts required to induce immobility differed ($F_{3,47} = 4.30$, $p = 0.009$, see Table 3.2). Fewer attempts were required to induce immobility in hens from pen one, when compared to pen two ($p = 0.001$) and pen four ($p = 0.017$), with a trend towards a difference from pen three ($p = 0.066$). Controlling for pen as a random factor, duration of the TI response did not differ with the number of attempts to induce it ($F_{2,44.9} = 0.92$, $p = 0.406$).

Table 3.2. Descriptive statistics for durations of tonic immobility and number of attempts required to induce the state for hens from the four experimental pens. Censored times had the maximum duration of 300 seconds.

	n	Median (s)	IQR (s)	Range (s)	*n* censored	Mean Attempts
Pen 1	17	269.3	226.4	12.3 - 300.0	7	1.27
Pen 2	10	290.0	155.4	60.1 - 300.0	5	2.30
Pen 3	11	261.4	172.5	78.0 - 300.0	4	1.82
Pen 4	15	109.7	282.8	6.5 - 300.0	3	1.93

In terms of ranging behaviour, proportional time spent in the barn ($F_{3,49} = 1.52$, $p = 0.222$) and wintergarden ($F_{3,49} = 1.53$, $p = 0.219$) did not differ between pens. However, pens differed in the proportional time that hens spent in the stone yard ($F_{3,49} = 3.39$, $p = 0.025$, see Figure 3.1). Hens in pen two (M = 13.81, SEM = 3.81) spent longer in the stone yard than hens in pen three (M = 4.72, SEM = 1.74; $p = 0.006$) and pen four (M = 5.97, SEM = 1.44; $p = 0.010$), with a trend towards longer times than pen one (M = 8.81, SEM = 1.32; $p = 0.088$). There was a trend towards differing proportional times spent on the range between pens ($F_{3,49} = 2.37$, $p = 0.082$). Time spent on the range was higher for hens from pen one (M = 9.53, SEM = 1.71) than pens two (M = 3.40, SEM = 2.22; $p = 0.034$) and three (M = 3.44, SEM = 2.12; $p = 0.030$). Hens from pen four spent an intermediate amount of time on the range (M = 5.80, SEM = 1.82),

which did not differ from the other three pens. Accounting for pen as a random factor and attempts needed to induce TI as a fixed factor, the duration of TI was not associated with time in any of the four areas (barn: $F_{1,46.8} = 1.69$, $p = 0.200$; winter-garden: $F_{1,46.3} = 0.58$, $p = 0.449$; stone yard: $F_{1,43.1} = 2.94$, $p = 0.094$; range: $F_{1,46.2} = 0.59$, $p = 0.448$).

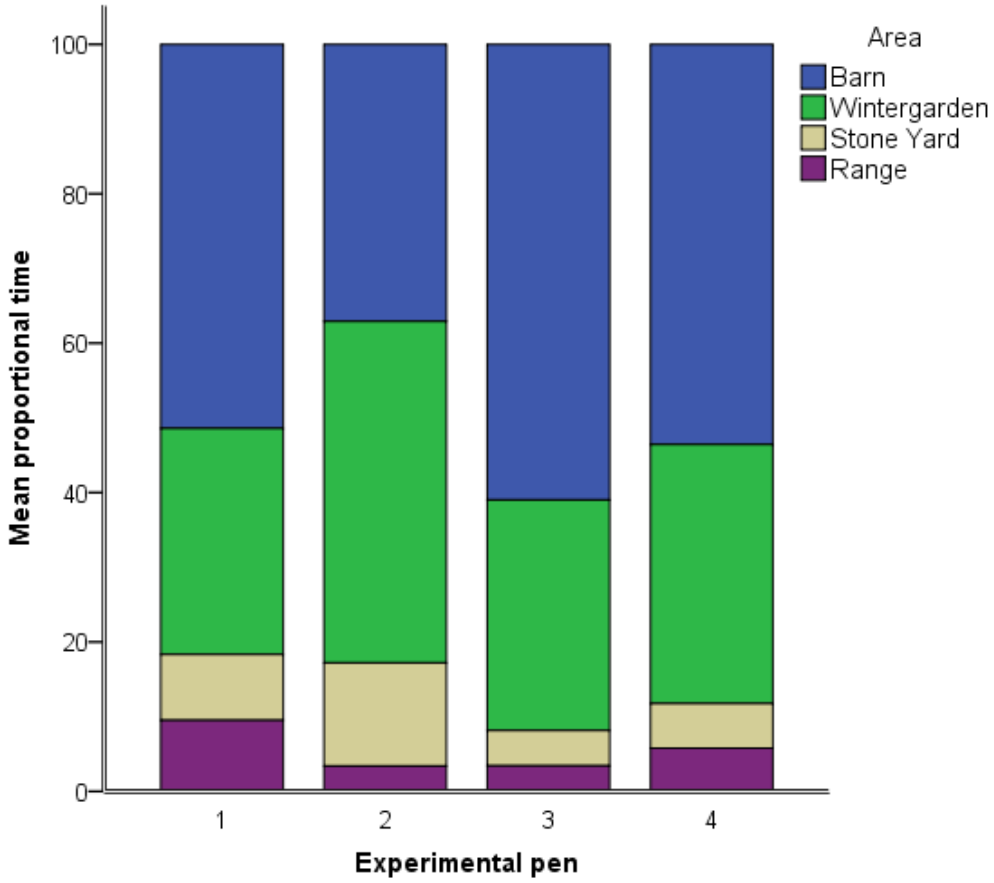

Figure 3.1. Mean proportions of available tracked time spent in each of the four areas by hens in the four experimental pens.

3.4.2 Hippocampal gene expression

As expected, expression of *LBR* mRNA covaried with expression of *PCNA* ($F_{1,88.5} \geq 264.7$, $p < 0.001$) and *DCX* ($F_{1,75.8} \geq 354.4$, $p < 0.001$) over all models. Samples of the same HF subregion taken from the two hemispheres did not differ from each other in expression of either gene (*PCNA*: $F_{1,45.4} \leq 0.296$, $p \geq 0.589$; *DCX*: $F_{1.47.5} \leq 0.114$, $p \geq 0.738$). Expression also did not differ between the rostral and caudal HF subregions for *PCNA* ($F_{1,62.0} \leq 1.72$, $p \geq 0.194$) or *DCX* ($F_{1,51.3} \leq 1.97$, $p \geq 0.166$) mRNA in any models.

3.4.3 Ranging & hippocampal gene expression

Proportional time spent in the barn did not correlate with *PCNA* mRNA expression across the whole HF ($F_{1,48.0} = 1.59$, $p = 0.214$), though there was a trend towards an interaction with HF subregion ($F_{1,45.7} = 3.38$, $p = 0.073$). In the rostral HF, there was a trend towards a negative

relationship between time in the barn and *PCNA* expression (B = -0.0030, SEM = 0.002, $F_{1,47.2}$ = 3.17, p = 0.082), with no relationship in the caudal HF (B = 0.0005, SEM = 0.001, $F_{1,40.2}$ = 0.33, p = 0.570). *PCNA* expression was not associated with proportional time spent in the winter-garden ($F_{1,47.5}$ = 0.03, p = 0.866), and there was no interaction with subregion ($F_{1,46.1}$ = 0.72, p = 0.401).

Proportional time spent in the stone yard positively correlated with *PCNA* expression over the whole HF ($F_{1,46.6}$ = 6.54, p = 0.014, B = 0.0018, SEM = 0.003), and there was an interaction with subregion ($F_{1,44.7}$ = 4.57, p = 0.038). While time in the stone yard positively correlated with *PCNA* mRNA in the rostral HF (B = 0.0146, SEM = 0.006, $F_{1,46.6}$ = 6.46, p = 0.014), there was no relationship in the caudal subregion (B = 0.0010, SEM = 0.033, $F_{1,34.4}$ = 0.12, p = 0.729).

The percentage of available time spent on the grassy range by hens did not correlate with their expression of *PCNA* mRNA in the HF as a whole ($F_{1,50.0}$ = 2.95, p = 0.092), but there was an interaction with HF subregion ($F_{1,46.2}$ = 5.10, p = 0.029, Figure 3.2A). Time on the range was positively associated with *PCNA* expression in the rostral HF (B = 0.0123, SEM = 0.006, $F_{1,45.6}$ = 4.10, p = 0.049) but not the caudal region (B = -0.0021, SEM = 0.003, $F_{1,41.8}$ = 0.46, p = 0.501). As time in the stone yard and time on the grassy range correlated positively with each other and both related to *PCNA* expression, an association between their combined values (i.e. the total available time spent outdoors) and *PCNA* levels was also explored. Time outdoors was related to *PCNA* expression ($F_{1,50.6}$ = 6.75, p = 0.012, B = 0.0004, SEM = 0.002) and an interaction ($F_{1,44.7}$ = 6.64, p = 0.013) indicated that this association was again attributable to a positive relationship in the rostral HF ($F_{1,46.0}$ = 7.36, p = 0.009, B = 0.0092, SEM = 0.003), with no correlation in the caudal subregion ($F_{1,39.0}$ = 0.02, p = 0.895, B = - 0.0002, SEM = 0.002).

Similarly, time spent on the grassy range did not co-vary with hippocampal *DCX* expression over the whole HF ($F_{1,51.4}$ = 0.56, p = 0.456) but the difference in slopes between the rostral and caudal subregions was significant ($F_{1,54.8}$ = 4.72, p = 0.034, Figure 3.2B). Time spent on the range was not associated with *DCX* expression in the rostral HF (B = 0.0062, SEM = 0.070, $F_{1,49.1}$ = 1.12, p = 0.296) but negatively correlated with *DCX* expression in the caudal HF (B = -0.0140, SEM = 0.007, $F_{1,53.2}$ = 4.09, p = 0.048). *DCX* expression was not associated with time in any other area (barn: $F_{1,49.1}$ = 0.02, p = 0.896; winter-garden: $F_{1,49.2}$ = 0.11, p = 0.744; stone yard: $F_{1,50.3}$ = 1.25, p = 0.270), nor did these parameters interact with HF subregion (time in barn*subregion: $F_{1,51.0}$ = 0.17, p = 0.679; time in winter-garden*subregion: $F_{1,51.1}$ = 0.26, p = 0.613; time in stone yard*subregion: $F_{1,50.9}$ = 0.96, p = 0.332).

Lastly, the mean number of daily transitions between the four areas made by individual hens (a crude proxy for activity levels) did not co-vary with expression of hippocampal *PCNA* ($F_{1,47.1}$ = 1.25, p = 0.261), though there was a trend towards an interaction with subregion ($F_{1,44.7}$ = 3.22, p = 0.080). The slope of the relationship was positive in the rostral HF (B = 0.0025, SEM = 0.002, $F_{1,45.7}$ = 1.64, p = 0.206), and negative in the caudal HF (B = 0.0007, SEM = 0.001, $F_{1,41.0}$ = 0.60, p = 0.443). *DCX* expression was not associated with number of transitions ($F_{1,49.0}$ =0.01, p = 0.915; transitions*subregion $F_{1,51.0}$ = 0.03, p = 0.855).

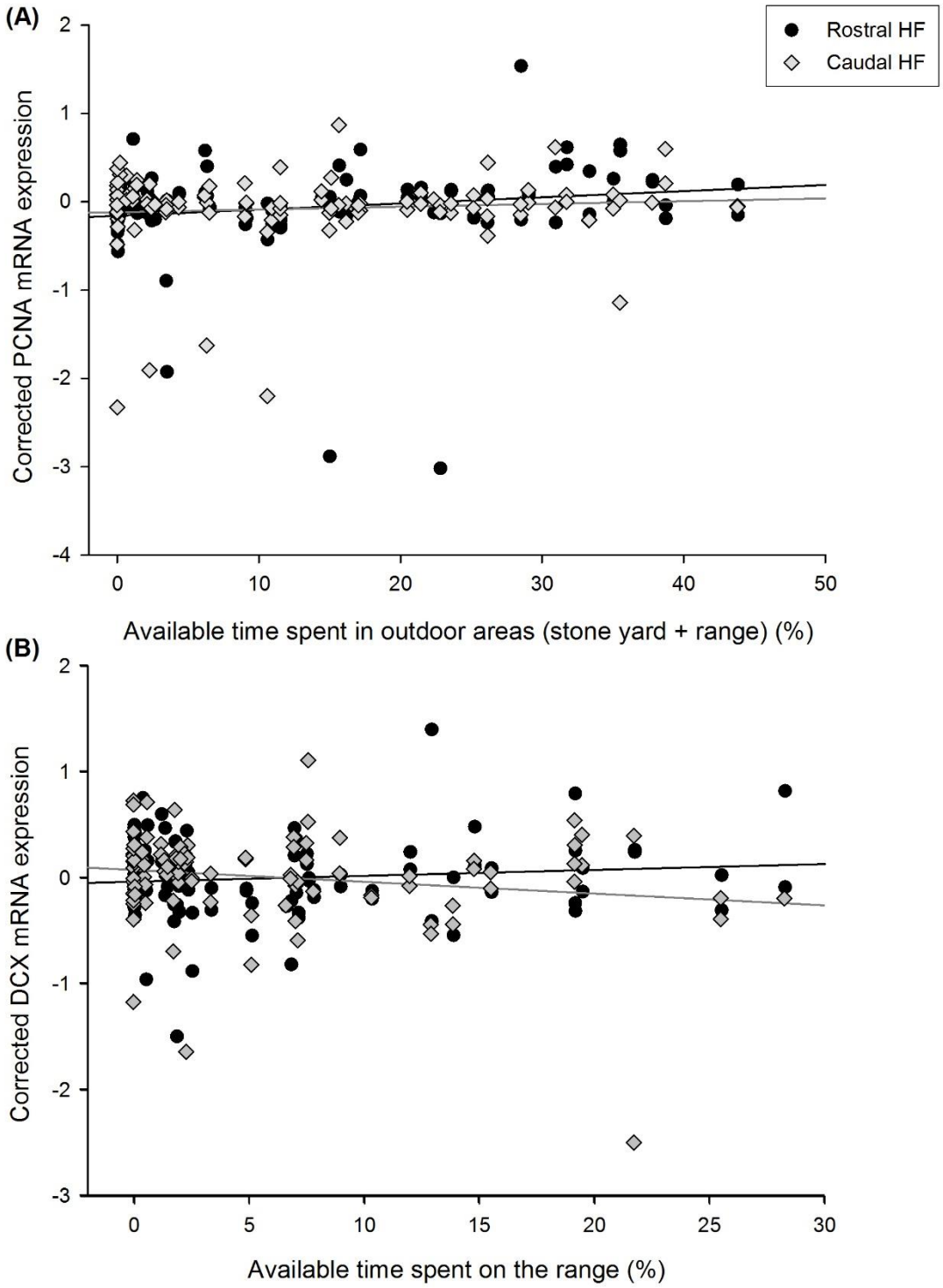

Figure 3.2. Relationships between the proportions of available time spent in outdoor areas by individual hens and corrected gene expression in the rostral and caudal subregions of the hippocampal formation. A) *PCNA* expression in relation to the total percentage of time spent in outdoor areas (i.e. the stone yard + grassy range) by focal hens. B) Doublecortin (*DCX*) expression in relation to the percentage of time that focal hens spent on the grassy range. Gene expression values are unstandardized residuals following correction for *LBR* expression, rostrocaudal subregion, sample and pen, in linear mixed models.

3.4.4 Tonic immobility & AHN

Duration of TI positively correlated with expression of *PCNA* over the whole HF ($F_{1,45.0} = 5.60$, $p = 0.022$) and did not interact with HF subregion ($F_{1,40.9} = 0.43$, $p = 0.516$, Figure 3.3A). *PCNA* expression did not differ between hens requiring one, two or three attempts to induce TI ($F_{2,39.8} = 1.46$, $p = 0.245$), nor did number of attempts interact with subregion ($F_{2,40.5} = 0.83$, $p = 0.443$). Conversely, hippocampal *DCX* mRNA expression was not correlated with TI duration ($F_{1,45.7} = 0.84$, $p = 0.365$) and there was no interaction with HF subregion ($F_{1,47.5} = 1.47$, $p = 0.231$, p = 0.333, Figure 3.3B). *DCX* expression did not differ with attempts to induce TI ($F_{2,46.6} = 0.97$, $p = 0.386$), and there was no interaction with subregion ($F_{2,46.6} = 1.47$, $p = 0.241$).

To verify that proportions of time spent outdoors and durations of TI explained independent proportions of the variance in *PCNA* expression, they were included as covariates in the same model. TI duration continued to co-vary with *PCNA* expression throughout the HF ($F_{1,43.2} = 4.39$, $p = 0.042$), and *PCNA* expression did not differ with the number of induction attempts ($F_{2,39.5} = 1.34$, $p = 0.273$). Proportional time in outdoor areas co-varied with overall *PCNA* expression ($F_{1,47.0} = 5.72$, $p = 0.021$), and the interaction between time outdoors and HF subregion remained significant ($F_{1,42.1} = 6.01$, $p = 0.018$).

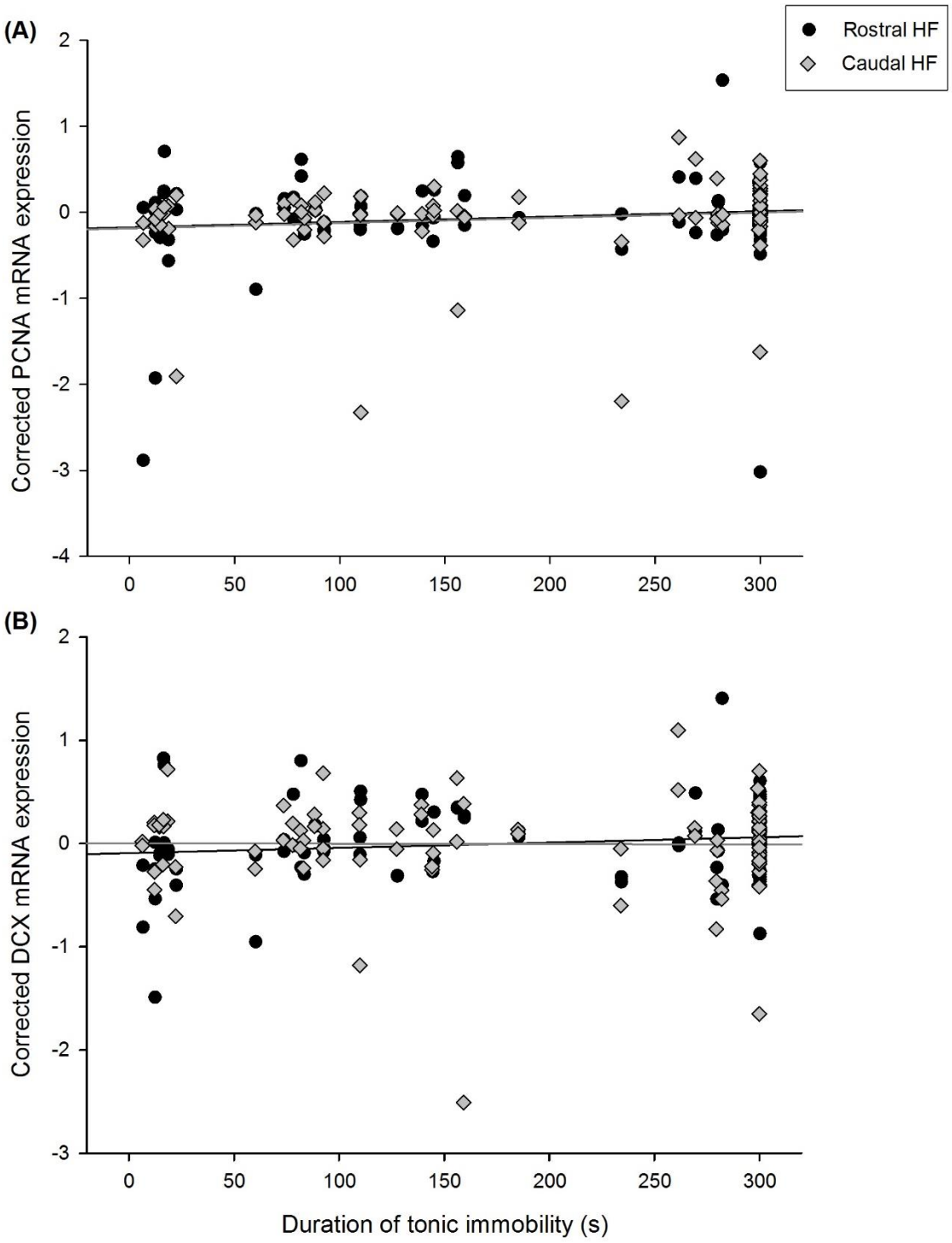

Figure 3.3. Relationship between durations of tonic immobility (seconds) for individual hens and residual expression of a) *PCNA* and b) doublecortin (*DCX*) mRNA in rostral and caudal subregions of the hippocampal formation, after correcting for *LBR* expression, rostrocaudal subregion, sample and pen in linear mixed models.

3.5 Discussion

Within the sampled flock of laying hens, individual differences in durations of TI were not correlated with time spent in any area of the housing system. Differences in TI have previously been reported between ranging sub-groups (Grigor *et al.*, 1995; Hartcher *et al.*, 2016), but another RFID-tracking study also failed to observe a relationship at the individual level (Larsen *et al.*, 2018). In the present sample, TI and use of the outdoor areas each explained separate portions of the total variance in expression of proliferative marker *PCNA*. The findings therefore support the existence of two independent relationships that link these behaviours to proliferation in the HF, each partially consistent with the hypothesised mechanisms. Durations of TI positively correlated with *PCNA* expression over the whole HF, suggesting that fearful, reactive hens have a higher level of hippocampal proliferation. This association is consistent with the predicted relationship between AHN and coping style. However, reactive hens would be expected to be less exploratory, but the associations between proliferation in the rostral HF subregion and proportional time spent in the furthest areas from the barn (the outdoor stone yard and range) were also positive. These subregional relationships are therefore more consistent with the predicted stimulatory effect of ranging experience on hippocampal cell proliferation. *DCX* expression, indicative of neuronal differentiation, displayed a generally different pattern to cell proliferation: it negatively co-varied with proportion of time spent on the range, but only in the caudal HF. These findings are considered in more detail below.

3.5.1 Hippocampal gene expression and tonic immobility (coping style)

Both higher basal levels of hippocampal proliferation and longer durations of TI are traits characteristic of individuals exhibiting a reactive (or passive) behavioural strategy/coping style (Lemaire *et al.*, 2008; Vindas *et al.*, 2017). Individual differences in proliferation, but not survival, have been positively related to the degree of freezing versus locomotion displayed by rats in a novel environment (Lemaire *et al.*, 2008) and negatively related to time spent in the centre of an open field by mice (Garrett *et al.*, 2012). Supporting a causal contribution of new cells to reactivity, mice with experimentally-suppressed neurogenesis freeze less than wild-type mice when faced with a novel environment and stimulus during contextual fear conditioning (Drew *et al.*, 2010). Reactive individuals also display enhanced behavioural flexibility (Höglund *et al.*, 2017), and hippocampal neurogenesis is necessary for flexible behaviour during learning tasks (Garthe *et al.*, 2009; Burghardt *et al.*, 2012). It is theorised that adult-born neurons promote the erasure of previously learned associations, in order to minimise proactive interference and facilitate the acquisition of novel associations (Anacker and Hen, 2017). Adult-

born neurons have also been demonstrated to inhibit the activity of mature granule cells under conditions of novelty and anxiety (Drew *et al.*, 2016; Anacker *et al.*, 2018). As such, AHN may form part of the intrinsic mechanism which links individual differences in cognitive flexibility to those in behavioural responses to challenge. A higher level of proliferation may translate to a relatively higher number of surviving new neurons under certain conditions (Fabel *et al.*, 2009), but current research does not indicate how proliferating cells may exert functional effects prior to maturation and integration. A corresponding relationship between TI and expression of *DCX* would therefore be expected, and the absence of such a correlation may relate to the influence of environmental factors on neuronal differentiation, or to a methodological explanation, each discussed further below. Furthermore, though reactive individuals are often less exploratory (Benus *et al.*, 1990; Hall *et al.*, 2015), no relationship between freezing (TI) and ranging existed for the sampled flock. Previous studies in hens have explicitly linked behavioural flexibility, but not fearfulness, to ranging tendencies (Ferreira *et al.*, 2019; Ferreira *et al.*, 2020b). It may be that other dimensions of personality, such as sociability (Ferreira *et al.*, 2020a), are also influential determinants of ranging behaviour, and obscure a simple relationship with reactivity.

3.5.2 Ranging experience & PCNA expression

As TI did not correlate with ranging in terms of the relative time spent in any area (internal or external), relationships between ranging behaviour and AHN are unlikely to relate to coping style, and may instead reflect the influence of ranging experience on hippocampal plasticity. In the rostral HF, time spent in both outdoor areas (the stone yard and grassy range) was positively associated with expression of *PCNA*. This relationship may be attributable to the stimulatory effect of factors including environmental complexity and exercise on hippocampal proliferation, as such experiences have been observed to preferentially modulate AHN in the septal rodent HF (Tanti *et al.*, 2012; Nishijima *et al.*, 2013; Tanti *et al.*, 2013; Ramirez-Rodriguez *et al.*, 2014). While the multi-tier barn interior comprises a complex, three-dimensional environment, all hens necessarily spent a substantial proportion of their time there: during the night and at other times when the additional areas were closed. Moreover, individual hens remained within the barn for a minimum of ~12% of the time that all areas were open, and it was the only location wherein certain key resources, including feed and nest boxes, were provided. Therefore, while the barn interior likely already comprised a cognitively challenging environment that could be considered enriched, this experience was shared by all birds. The winter-garden also provided resources in the form of drinkers and perches, and perhaps represented an extension of the barn in that it was used by all but one hen. In contrast, the lower

proportion of hens that also regularly ventured farther afield, into the uncovered stone yard and range, effectively had a larger home range. This may entail maintenance of a larger mental map, while presenting greater navigational challenge to return to the resources provided inside. Size of the home range positively predicts hippocampal proliferation across species of rodent (reviewed in Konefal *et al.* (2013), and the variety of territory coverage by individual mice roaming a complex home environment was strongly correlated with AHN (Freund *et al.*, 2013). In birds, AHN rates are higher in migratory than non-migratory subspecies (of white crowned sparrow, *Zonotrichia leucophrys*) (LaDage *et al.*, 2010b) and are stimulated by spatial-cognitive demand in experimental settings (Patel *et al.*, 1997; LaDage *et al.*, 2010a). An increase in HF proliferation was observed following the storage and retrieval of food caches by Marsh tits (*Poecile palustris*) (Patel *et al.*, 1997), making the spatial-cognitive challenge of ranging outside a likely contributor to the observed relationship with *PCNA* expression. While environmental enrichment has been found to upregulate numbers of proliferating cells in mice (Kempermann *et al.*, 1998; Steiner *et al.*, 2008; Tanti *et al.*, 2012; Tanti *et al.*, 2013), physical activity is perhaps a more robust driver of expansion of the precursor cell pool (Van Praag *et al.*, 1999; Fabel *et al.*, 2009). There was a trend for the number of transitions that hens made between areas to correlate with rostral *PCNA* expression, which may also point to a similar relationship between exercise and proliferation in chickens. However, as it was not possible to measure the individual distances travelled within each area, this measure provides only a crude proxy, and future experimental work will be needed to establish such an association.

3.5.3 Ranging experience and DCX expression

Given that the stimulation of proliferation by a positive experience such as exercise leads to a subsequent increase in the number of surviving new-born neurons (Fabel *et al.*, 2009), the lack of corresponding positive relationships between time in the outdoor areas and rostral expression of *DCX* is also surprising. While *PCNA* is a non-specific marker of mitosis, around 80% of surviving proliferating cells in the adult rat DG are estimated to differentiate into mature neurons, compared to 15% that become astroglia (Lee *et al.*, 2006). Moreover, around 30% of the population of DCX^+ cells in the adult mouse DG are estimated to be proliferative (type-2b and -3 progenitors) (Plümpe *et al.*, 2006). This finding is also at odds with the robust effect of enrichment on later stages of AHN e.g. (Kempermann *et al.*, 1997; Van Praag *et al.*, 1999). Moreover, in the caudal HF, time spent on the grassy range alone correlated negatively with *DCX* expression. Downregulation of AHN consistently occurs following the experience of stress, and the temporal hippocampus in rodents (Hawley and Leasure, 2012; Lehmann *et al.*, 2013) and the caudal HF in laying hens (Gualtieri *et al.*, 2019) are known to be particularly

sensitive. As certain forms of stress have a greater negative influence on the later survival of young neurons (Lee *et al.*, 2006; Van Bokhoven *et al.*, 2011; Castilla-Ortega *et al.*, 2014), it is possible that this factor is responsible for the decoupling of relationships with *PCNA* and *DCX* expression. The observed negative correlation between time on the range and caudal *DCX* expression implies that, while outdoor visits provide further environmental complexity (and possibly exercise), hens that spend more time on the range also experience more stress. This association is perhaps related to the consistent finding that many hens provided with outdoor access choose not to range (Richards *et al.*, 2011; Gebhardt-Henrich *et al.*, 2014; Campbell *et al.*, 2017; Larsen *et al.*, 2017). Indeed, the general assumption that the range represents an exclusively positive environment has not been demonstrated.

There is some evidence to suggest that enrichment may be stressful even within controlled laboratory settings. One study found that housing mice in enriched cages including running wheels upregulated DCX immunoreactivity and mRNA expression in the septal mouse dentate gyrus, while suppressing levels in the temporal region (Gualtieri *et al.*, 2017). Housing domestic pigeons in an enriched environment has also been observed to increase the number of *DCX*-expressing neurons in the HF (septal/rostral and temporal/caudal HF were not distinguished), while simultaneously increasing average durations of TI (Melleu *et al.*, 2016). Though a group effect was observed, the authors found no correlation between TI times and cell numbers on an individual level. Further investigation into whether some general aspects of enrichment, perhaps relating to the cognitive challenge, are intrinsically associated with stress may therefore be warranted.

Beyond laboratory enrichment, ranging outdoors may expose hens to unpredictable sources of stress, such as adverse weather conditions and sightings of predators. Individual range use was previously positively correlated with the CORT response to handling and flightiness to avoid a human (Larsen *et al.*, 2018), which may indicate greater anxiety. As time in the stone yard was not negatively associated with *DCX* expression, the characteristics which distinguish the range itself may be particularly stressful. Both areas were uncovered, potentially exposing hens to rain and sightings of aerial predators, but weather conditions such as wind may be more salient on the large, open range than in the smaller, fenced stone yard. Contact with soil is also associated with exposure to a greater burden of parasites (Lay Jr. *et al.*, 2011), which may be a source of immune-stress. Perhaps due to extensive cover in the ancestral environment of red junglefowl, hens show a collective preference for shelter (Singh and Cowieson, 2013), whereas use of the open range entails being exposed. Over multiple commercial farms, the number of birds using an outside range correlates positively with the amount of tree cover provided

(Dawkins *et al.*, 2003), while addition of tree cover or shelters increases use of the range (Lubac and Mirabito, 2001; Zeltner and Hirt, 2008). The range in the present study was relatively barren and did not contain trees or other forms of shelter. Consistent with the sampled hens spending less time on the range than in other areas, nearest-neighbour-distance is generally observed to increase with increasing distance from the barn (Chielo *et al.*, 2016). This lack of proximity to conspecifics may be stressful, due to greater perceived predation risk or social isolation. On the other hand, as frequently ranging hens choose to be less close to their conspecifics (Ferreira *et al.*, 2020a), it is also possible that hens visit the range to escape social conflict with flock mates. In this case, the experience of stress would drive visits outside. However, we might therefore also expect to see an association with coping style, meaning this explanation is probably not consistent with the lack of correlation between ranging and TI.

In mice, survival to the point of maturation may be promoted specifically within the septal hippocampus by environmental enrichment (Tanti *et al.*, 2012), whereas neuronal differentiation may be suppressed over the whole hippocampus by certain paradigms of chronic stress (Nollet *et al.*, 2012; Tanti *et al.*, 2013). If spending time outside is such an experience for hens, then the stimulatory influence of cognitive stimulation may have counteracted the effect of stress in the rostral subregion, leaving an observable negative relationship only at the caudal pole. In mice, a combination of experimental stress and cognitive stimulation in the form of maze learning similarly led to a preferential reduction in AHN in the temporal subregion (Hawley *et al.*, 2012). Such an interaction could explain the lack of a positive relationship between time spent outside and rostral *DCX* expression. In the case of proliferation, environmental enrichment has been found to elevate levels specifically within the septal mouse hippocampus in some instances (Tanti *et al.*, 2013), but mediate an increase in both subregions in others (Tanti *et al.*, 2012). Proliferating cell numbers may be reduced most severely in the temporal subregion by chronic stress (Hawley and Leasure, 2012), or be suppressed uniformly (Tanti *et al.*, 2013). It is therefore difficult to conclude whether an influence of stress on proliferation in the caudal HF may have contributed to the rostral-specific nature of the association between outdoor ranging and PCNA expression in the present study.

3.5.4 Methodological considerations

It is important to note that, while transcription of the *DCX* gene has been demonstrated to be a valid proxy for the effects of running on neuronal differentiation in mice (Gualtieri *et al.*, 2017), this association has yet to be verified in birds. Quantification of both *DCX* mRNA and DCX-immunoreactive cell counts in hippocampal tissue from the same sample of hens will be an

important future step to validate the present methodology. Unlike in the mammalian brain, adult neurogenesis is not restricted to a single subdivision of the avian HF (equivalent to the dentate gyrus), meaning it is not possible to micro-dissect a particular substructure or to use a control gene specific to its cellular population (as with Prox1 for granule cells) for normalisation. The small effect sizes observed may reflect a complex interaction between the multiple internal and external factors which relate to AHN, but could also relate to a methodological issue, such as post-transcriptional processes which complicate the relationship between mRNA and protein levels (Liu *et al.*, 2016). Previous research has noted background expression of *DCX* mRNA in non-neurogenic subdivisions of the mouse hippocampus, with levels unresponsive to running exercise (Kremer *et al.*, 2013). Though the majority of the avian telencephalon is neurogenic, low-level transcription of *DCX* in other types of HF cell, such as mature neurons undergoing dendrite-remodelling (Nacher *et al.*, 2001), might obscure correlations with expression of the marker by differentiating immature neurons. This issue of background expression relates specifically to the use of *DCX* as a marker, meaning our results for *PCNA* expression may be more reliable. Future comparison of the qPCR method and standard morphological techniques to quantify neurogenesis may indicate whether decoupling of *PCNA* and *DCX* expression here is more likely to reflect differential effects of stress, or to have a methodological explanation.

3.5.5 Conclusions

To conclude, individual differences in time spent in outdoor areas and durations of TI in a commercial laying hen flock both positively correlated with cell proliferation in the HF (time outdoors only in the rostral half) but were not related to each other. As found in other species, reactive hens had higher basal *PCNA* expression in the hippocampus, while environmental enrichment and exercise through ranging were positively associated with *PCNA* expression in the rostral HF. Hippocampal proliferation thus most likely reflects both personality in terms of behavioural strategy/coping style and the influence of experience. On the other hand, expression of neuronal differentiation marker *DCX* in the caudal HF was negatively related to ranging experience. As the caudal HF may be preferentially sensitive to stress, it is possible that some aspects of ranging are both stimulating and stressful at the same time. Overall, individual differences in laying hen behaviour are reflected in hippocampal plasticity, but the relationship between use of outdoor areas and affective experience may be mixed, while interactions with intrinsic factors are likely complex.

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Chapter 4. Severity of Keel Bone Damage Negatively Predicts Adult Neurogenesis in the Laying Hen Hippocampal Formation

4.1 Abstract

In commercial flocks of laying hens, keel bone fractures (KBFs) are prevalent and associated with signs of poor welfare, including behavioural indicators of pain. However, whether their impact is severe enough to induce a depressive-like state of chronic stress is unknown. As levels of adult hippocampal neurogenesis (AHN) in the brains of mammals and birds are suppressed by the chronic experience of stress, this measure may be applicable as a neural biomarker of subjective welfare state. Radiographs obtained longitudinally from Lohmann Brown laying hens housed in a commercial multi-tier aviary were used to score the severity of naturally occurring KBFs between the ages of 21 and 62 weeks. Individual mobility data was collected using an infrared tracking system to record transitions between aviary zones. Focal hens with severe KBFs at 3-4 weeks prior to sampling (n=15) had lower densities of immature doublecortin-positive (DCX^+) multipolar and bipolar neurons in the hippocampal formation (HF) than focal hens with minimal fractures (n=9). KBF severity scores at this time also negatively predicted DCX^+ cell numbers across the whole HF on an individual level, while the duration of time since a hen had acquired their first fracture tended to negatively correlate with numbers of DCX^+ neurons in the caudal HF. Activity levels 3-4 weeks prior to sampling were not associated with AHN. KBFs thus appear to present a source for chronic stress to laying hens, inducing a negative affective state that lasts at least 3-4 weeks. Management steps to reduce their occurrence are therefore likely to have significant benefits for welfare.

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4.2 Introduction

Keel bone fractures (KBFs) present a serious welfare problem for the egg production industry, given that between 20 to 96% of birds within commercial flocks in various countries are reported to have some level of damage (Belgium: Heerkens *et al.* (2013); Canada: Petrik *et al.* (2015); Denmark: Riber and Hinrichsen (2016); The Netherlands: Rodenburg *et al.* (2008a); Switzerland: Kappeli *et al.* (2011), Stratmann *et al.* (2015); and the UK: Wilkins *et al.* (2011), Tarlton *et al.* (2013), Toscano *et al.* (2015)). Estimates of KBF prevalence increase with age, rising from 5.5% of birds affected within a flock at onset of lay (Richards *et al.*, 2012) to as many as 97% by the end of a production cycle (Rodenburg *et al.*, 2008b). Many KBFs appear to arise from collisions, both with perches (Sandilands *et al.*, 2009), which hens are highly motivated to use (Weeks and Nicol, 2006), and other equipment including drinkers and support beams. Although the presence of a KBF does not suppress perching behaviour (Gebhardt-Henrich and Fröhlich, 2015), it does reduce frequency of range access via popholes (Richards *et al.*, 2011). Further behavioural evidence also supports the assumption that KBFs are a source of pain or discomfort for laying hens (reviewed by Riber *et al.*, 2018), with recent studies demonstrating their association with altered movement throughout the aviary (Rentsch *et al.*, 2019; Rufener *et al.*, 2019). Focusing on more direct assessments of movement and pain, hens with KBFs display greater latencies to fly down from perches 100 and 150 cm above the floor to obtain a food reward than hens without fractures, whilst delays in fractured birds alone are reduced following administration of various analgesics (Nasr *et al.*, 2012a; Nasr *et al.*, 2012b; Nasr *et al.*, 2015). Furthermore, a conditioned place preference for the location in which the analgesic was administered is observed to develop only in hens with KBFs (Nasr *et al.*, 2013). While this indicates that short-term relief from pain arising from keel fractures is a reinforcing occurrence, whether their un-medicated experience is negative and/or salient enough to produce a chronic state of stress is unknown. Furthermore, whether pain arising from KBFs has an affective component for chickens, in addition to sensory/nociceptive components, has yet to be explored.

In mammals, the process of adult hippocampal neurogenesis (AHN) is sensitive to the valence of prolonged experiences (Warner-Schmidt and Duman, 2006; O'Leary and Cryan, 2014), and is downregulated following chronic exposure to stress (Gould *et al.*, 1998; Tanti *et al.*, 2012; Miller and Hen, 2015), often especially in the temporal hippocampus (Jayatissa *et al.*, 2006; Elizalde *et al.*, 2010; Perera *et al.*, 2011; Hawley and Leasure, 2012; Nollet *et al.*, 2012; Tanti *et al.*, 2013). Evidence further supports the application of AHN as a proxy for the emotional component of stress, as levels of $BrdU^{+}$ cells in the temporal DG are restored by an

antidepressant agent only in individual rats that also show behavioural recovery from anhedonia (Jayatissa *et al.*, 2006). Models of chronic neuropathic pain in rats and mice have no influence on baseline plasma CORT titres (Dellarole *et al.*, 2014; Dimitrov *et al.*, 2014; Romero-Grimaldi *et al.*, 2015), but induce measurable chronic stress in the brain, reflected by marked suppression at several stages of AHN. Up to 35 days after various forms of neuropathic injury, significant reductions in hippocampal numbers of Ki-67 (Romero-Grimaldi *et al.*, 2015) and $PCNA^+$ (Dimitrov *et al.*, 2014) proliferating cells, doublecortin (DCX)-positive differentiating neurons (Mutso *et al.*, 2012; Dimitrov *et al.*, 2014) and $BrdU^+$ post-mitotic cells (surviving four weeks) (Romero-Grimaldi *et al.*, 2015) have been reported in comparison to sham-operated animals. Inflammatory pain similarly decreased the number of $BrdU^+/DCX^+$ co-labelled neurons when the duration was chronic (10-14 days), but not acute (3-7 days) (Zheng *et al.*, 2017). Pain-induced downregulation of AHN is accompanied by behavioural indicators of anxiety- and depressive-like states, including greater immobility in the FST, avoidance of open maze arms and the centre of an open field, longer durations of NSF and latencies to leave a dark box, an inability to extinguish contextual fear, and impaired novel object recognition (Mutso *et al.*, 2012; Dimitrov *et al.*, 2014; Zheng *et al.*, 2017). The magnitude of the pain stimulus employed may have a corresponding quantitative effect on neuronal differentiation, as DCX expression compared to sham mice was reduced by 60% following partial sciatic nerve ligation, and by 33% following introduction of a looser sciatic cuff (Dimitrov *et al.*, 2014). Moreover, these alterations in neurogenesis and affective behaviour persisted for at least three weeks beyond surgical reversal of the injury (Dimitrov *et al.*, 2014), demonstrating their independence from on-going nociceptive stimulation. Chronic stress arising from experience of the pain stimulus may thus have been associated with induction of a prolonged negative subjective state.

Accumulating evidence suggests that AHN levels in birds are similarly downregulated by chronic exposure to stress (Barnea and Nottebohm, 1994; LaDage *et al.*, 2010; Robertson *et al.*, 2017; Taufique *et al.*, 2018), while the caudal HF subregion (putatively homologous to the temporal mammalian hippocampus (Smulders, 2017)) may be preferentially sensitive in chickens (Gualtieri *et al.*, 2019). If KBFs in laying hens present a pain stimulus comparable to those induced in the aforementioned rodent models, then individual differences in acquired severity within commercial housing units may translate to varying degrees of chronic stress for hens. This has clear implications for welfare management. To test this hypothesis, we sampled hens at two extremes of the KBF spectrum within a commercially relevant aviary housing system, namely those with minimal and severe fractures, whilst collecting longitudinal data on individual fracture severity scores. To quantify the number of surviving new-born cells

generated through AHN, we stained sections for DCX, an endogenous protein marker of migratory immature neurons in the mammalian (Couillard-Despres *et al.*, 2005) and avian (Balthazart *et al.*, 2008) brain. We predicted that hens with severe KBFs would have a lower density of DCX^+ cells in the HF than counterparts with minimal KBFs, with the effect potentially more pronounced at the caudal HF pole. In addition, a linear relationship across hens was predicted, whereby individuals with greater KBF severity scores were expected to have a relatively lower density of new-born hippocampal cells. Expression of *DCX* mRNA was also quantified in HF tissue from the same sample of hens, to determine whether this molecular marker displayed comparable sensitivity in relation to KBF severity. Finally, since pain arising from KBFs might be expected to reduce hen's mobility, and exercise is known to linearly predict AHN in rodents (Allen *et al.*, 2001), it was necessary to account for a potential confounding effect of activity on AHN levels. An infra-red tracking system was thus used to record the number of transitions that individual hens made between aviary zones. Running has a sustained effect on new-born cell numbers in the mouse hippocampus five weeks after it is discontinued (Fabel *et al.*, 2009), and DCX is expressed for approximately four weeks from the start of neuronal differentiation (Balthazart *et al.*, 2008). As such, whether hens' activity levels ~3-4 weeks previously were associated with DCX^+ cell counts - either independent of, or in relation to, KBF scores - was also explored.

4.3 Methods

4.3.1 Ethical statement

Ethical approval for the study was obtained from the Veterinary Office of the Canton of Bern in Switzerland (approval number BE31/15) and the the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #549). The experiment complied with Swiss regulations regarding the treatment of experimental animals.

4.3.2 Animals & housing

One day old Lohmann Brown (LB) and Lohmann Selected Leghorn (LSL) chicks arrived at the Aviforum research facility in Zollikofen, Switzerland, where they were housed within eight mixed hybrid rearing pens. The study reported here was part of a larger effort, the facilities and management protocols of which are described in full by Rufener *et al.* (2019). At 18 weeks of age, hens were transferred to a commercial aviary system for laying hens within a layer barn on site. The layer barn was divided into 20 identical pens (450 x 700 x 230 cm), each holding 225 hens. In each of three pens, 20 focal LB hens were co-housed with 205 LSL hens of the same age. The minority LB hens within each pen all came from the same rearing pen. The

differing phenotype of the white LSL hens facilitated the identification and catching of focal LB hens throughout the study. Three other pens in the barn contained focal LSL hens with a majority of LB hens, but as this strain was not sampled for quantification of AHN, their results are not reported here (see Rufener *et al.* (2019) for mobility data). In addition to spiral leg rings colour-coded for pen (Fieger AG, Untertuttwil, Switzerland), focal hens were marked with flexible legbands (Roxan Developments Ltd, Selkirk, United Kingdom) displaying individual identification numbers. Hens were left for three weeks to habituate to the new housing environment before mobility tracking commenced.

Each pen of the layer barn was equipped with a commercial aviary system (Bolegg Terrace, Vencomatic; Krieger AG, Ruswil, Switzerland). The system consisted of three tiers: 1) a lower tier (73 cm above ground), 2) a nest box tier with integrated group nests (153 cm above ground) and 3) a top tier (220 cm above ground), and was subject to minor modifications from the standard installed model. Specifically, the perch on the top tier was removed and one drinker line was moved from in front of the nest box to the top tier. Food and water were provided *ad libitum* through automatic feeding chains and nipple drinkers on the top and lower tier (with feed refreshed every two hours during light hours). Artificial light was provided from 02:00 h until 17:00 h, with a 5-minute dawn (02:00 h – 02:05 h) and a 30-minute dusk (16:30 h – 17:00 h) phase. Natural daylight was managed via curtains in front of the windows, which were open from 08:00 h until 16:00 h. Perches consisted of round metal rails (diameter: 3.2 cm, length: 230 cm) located on the top tier for roosting (six perches at 270 cm height, two perches at 300 cm height) and across the system to facilitate movement between tiers (three perches at 190 cm and 125 cm above ground on each side of the aviary, on top of the feeder on the lower tier). In total, 14 cm of perch space per hen was provided. The floor besides and underneath the aviary was covered with wood shavings (approx. 10 cm deep). Stocking density was 7.4 hens / m^2 of accessible floor space, which included the littered floor area and all mesh grid areas in the lower and top tiers. Pecking opportunities were provided in the form of autoclaved aerated concrete stones (Xella Porenbeton Schweiz AG, Zurich, Switzerland) and *ad libitum* mineralized pecking stones (FORS 228 Pickschale Geflügel; Kunz Kunath AG, Burgdorf, Switzerland). To increase opportunities for explorative behaviour (scratching, pecking), straw was supplied in racks placed in the litter area. The winter-garden provided a covered area (9.32 m^2) external to each pen, containing wood shavings and a dust bathing area filled with sand, and was accessible via popholes (15 cm above ground level) which opened automatically at 10:00 h and were closed manually between 16:00 and 16:30 h.

4.3.3 Data collection

Data were collected at 11 time points during the production cycle. For one pen (20 focal birds), this occurred when the birds were 21, 24, 27, 31, 35, 39, 44, 48, 52, 57, and 61 weeks of age. For practical reasons, data from the remaining two pens (40 focal birds) were collected the subsequent week, i.e., 22, 25, 28, 32, 36, 40, 45, 49, 53, 58, and 62 weeks of age. Data on individual mobility were collected for six days per time point, and on the final day, hens were radiographed to detect fractures.

4.3.4 Individual mobility

Individual mobility was recorded using a custom-made infrared (IR) tracking system which has been previously described and validated (Rufener *et al.*, 2018c). Infrared emitters were installed on the vertical grid panels dividing pens and generated infrared beams encoded with specific signals for the five pre-defined zones: litter, lower tier, nest box, upper tier, and winter-garden. Infrared receivers were mounted on the legbands of focal hens and recorded the zone-specific signals produced by the IR emitters with a frequency of 1 Hz, along with the date and time of each zone change. The system therefore recorded vertical transitions made across the aviary, but did not track horizontal movement within zones. Receivers were covered by a small plastic container to protect from dust, moisture and faeces (outer diameter: 3.1 cm, height: 2 cm; Semadeni AG, Ostermundigen, Switzerland) throughout the experiment. Containers were replaced if they became opaque due to dirt or scratches. Equipment mounted on the hens weighed 9.4 g, well below the suggested limit of 5% of body mass (Siegford *et al.*, 2016).

At each of the 11 study time points, hens were caught and equipped with IR receivers on the day preceding mobility data collection (day 1). This allowed time for both habituation to the additional weight of the receiver and re-establishment of normal mobility behaviour after handling, given this experience has been shown to impact tonic immobility behaviour occurring directly afterwards (Jones, 1992). Monitoring devices have been found to reduce exploration by adult hens on the day of fitting, but to have negligible effects on behaviour from two days onwards (Buijs *et al.*, 2018). Mobility data were therefore collected from day 2 to day 7, before receivers were removed and data were downloaded as CSV files on day 8.

4.3.5 Keel bone assessment/fracture severity scoring

At each time point, after removing the IR receiver on day 8, hens were radiographed to detect keel bone fractures using a mobile X-ray unit (GIERTH HF 200 ML; x-ray tube Toshiba D-124 with maximal acceleration voltage of 100kV; x-ray plate Canon CXDI-50G; software Canon

CXDI Control Software NE; distance: 80 cm, voltage: 46 kV / 2.4 mAs). Hens were firmly held by both legs, carefully turned upside down and fixated in padded metal shackles to induce immobility (Širovnik and Toscano, 2017). The procedure took approximately 10-20 seconds per radiograph and occurred 11 times (T1-T11) throughout each hen's lifetime. In a previous study, KBF severity at 61 weeks did not differ between repeatedly-radiographed focal hens and non-focal hens radiographed only at this time, indicating that multiple radiographs had no negative impact on keel integrity (Rufener *et al.*, 2018a). Though focal LSL hens produced fewer eggs, it is not clear if this related to stress of the radiograph procedure or of being the minority hybrid, whilst egg production in focal LB hens (used in the present study) was not affected (Rufener *et al.*, 2018a).

Radiographs were imported to the PACS (Picture Archiving and Communication System; IMPAX EE, Agfa Healthcare, Bonn, Germany) of the Department of Clinical Radiology (Vetsuisse Faculty, University of Bern) as DICOM files. For subsequent analysis, radiographs were downloaded from the PACS as JPEG files.

Radiographs were analyzed according to aggregate fracture severity and the presence of a visible fracture gap. The observer was blind to both the age and identity of the hen. Aggregate fracture severity was assessed using a tagged visual analogue scale ranging from "no fracture" to "extremely severe", resulting in a continuous variable ranging from 0.0 to 10.0. The system and its validation is described in detail by Rufener *et al.* (2018b). Eleven days after final radiographs were conducted, preliminary analysis of processed radiographs up to the penultimate time point (T10) was used to select 12 birds with minimal KBFs and 12 birds with severe KBFs for brain sampling. This sample size previously afforded sufficient power to detect an effect of chronic stress on the same AHN outcome measure in laying hens (Gualtieri *et al.*, 2019). It was not possible to select a control group of hens with no fractures, as although these were very minor in some cases, birds entirely free of KBFs did not exist by the end of the study. The higher proportion of KBFs detected here compared to previous studies may relate to the sensitivity of the relatively novel radiography technique, compared to less reliable methods such as palpation (Rufener *et al.*, 2018b).

4.3.6 Tissue collection & processing

Following a delay of 4.5 (pen 1) or 3.5 (pens 2 & 3) weeks since the final x-ray, focal hens (n=24) were killed via an intravenous injection of pentobarbital (Esconarkon, 0.3 ml/hen). Immediately thereafter, brains were removed from the skull, placed into 0.1 M PBS in a Petri dish and divided along the longitudinal fissure with a scalpel. For each sampled hen, one

hemisphere was processed for immunohistochemistry, while the HF from the other hemisphere was allocated for molecular biology. To balance possible lateralisation of AHN and its responsivity, tissue collected for each purpose was alternated between the left and right hemisphere within the minimal and severe KBF groups. In preparation for molecular analysis, the HF was dissected from the forebrain and divided midway across the rostro-caudal axis to produce two subsamples (rostral & caudal) containing equal amounts of tissue. These isolated HF regions were placed in sample tubes containing 1.5 ml of RNAlater® Stabilization Solution (Thermo Fisher Scientific, UK) and refrigerated for 24 hours before storage at -30°C. The remaining hemisphere from each brain was immersion fixed for 44-48 h in 4% paraformaldehyde in 0.5 M Phosphate Buffered Saline (PFA - PBS) at 4°C in preparation for immunohistochemistry. Samples were then cryoprotected in a solution of 30% sucrose in 0.5 M PBS, before being embedded in OCT (4583, Electron Microscopy Sciences - USA). Coronal sections (50 μm) were cut on a cryostat (HM 550, Microm – Germany) and stored in cryoprotectant solution (30% glycerol, 30% ethylene glycol, 0.1M PBS) at -20^{o}C. Serial sections taken at 400μm intervals were processed for immunohistochemistry.

4.3.7 Immunohistochemistry

To quantify the number of surviving new-born cells generated through AHN, sections were stained for DCX, an endogenous protein marker of migratory immature neurons in the avian brain (Balthazart *et al.*, 2008). Staining was conducted over six batches, each containing tissue from four birds, balanced for KBF status. All washes were conducted in 0.1 M PBS for 3 x 5 min. Free-floating tissue slices were first washed to remove cryoprotectant, then underwent 30 minutes endogenous peroxidase inhibition in 1% H_2O_2 (Sigma-Aldrich, UK). Samples were washed again before 60 minutes incubation in blocking solution, containing 2% normal goat serum and 1% Bovine Serum Albumin [BSA] dissolved in 0.1 M PBS that contained 0.3 % Triton X-100. After a quick rinse in distilled H_2O, samples were incubated overnight in rabbit polyclonal to doublecortin primary antibody (Abcam Cat# ab18723, RRID:AB_732011) at concentration 1:1000 (4^{o}C). Following washes, samples were incubated for 120 minutes at room temperature in 1:500 biotinylated anti-rabbit IgG secondary antibody, made in goat (Vector Labs, BA-1000). Samples were washed before conjugate enzyme incubation in 1:250 Horse Radish Streptavidin (Vector Labs, SA-5004) for 60 minutes. Following washes in PBS and a wash in dH_2O (1 x 5 min), 30 seconds chromogen incubation in 3,3'-Diaminobenzidine (DAB) was conducted by diluting SIGMAFAST tablets in pure water to final concentration of 0.35mg/ml. Tissue was rinsed immediately in dH_2O to stop the reaction. Following final washes, slices were mounted onto gelatine-subbed slides using a paintbrush in dH_2O. Once dry,

slides were soaked for 2 x 5 minutes in Histoclear before coverslipping using Eukitt® (03989 FLUKA). Excess mounting medium was cleaned from the slides using a razor blade after drying.tttttttt

4.3.8 Quantification of AHN

A representative series of coronal sections bearing tissue from the rostral and caudal chicken HF is displayed in Figure 4.1. For every animal, 4 to 6 hippocampal sections 800 µm apart were systematically analysed, starting with the rostral-most section bearing hippocampal tissue. This sampling spanned roughly 1/16th of the rostral and 1/8th to 1/4th of the caudal HF, given the latter region curls around the back of the forebrain and is thus contained within fewer coronal slices. Hippocampal slices were analysed with an optical microscope (Leica DM6B-Z, Germany) equipped with a digital video camera (Leica DFC450 C, Germany) and motorized stage system (Leica AHM, Germany) to step through sections for systematic sampling. Quantification was performed by a single observer (EA), blind to the KBF status of the animals.

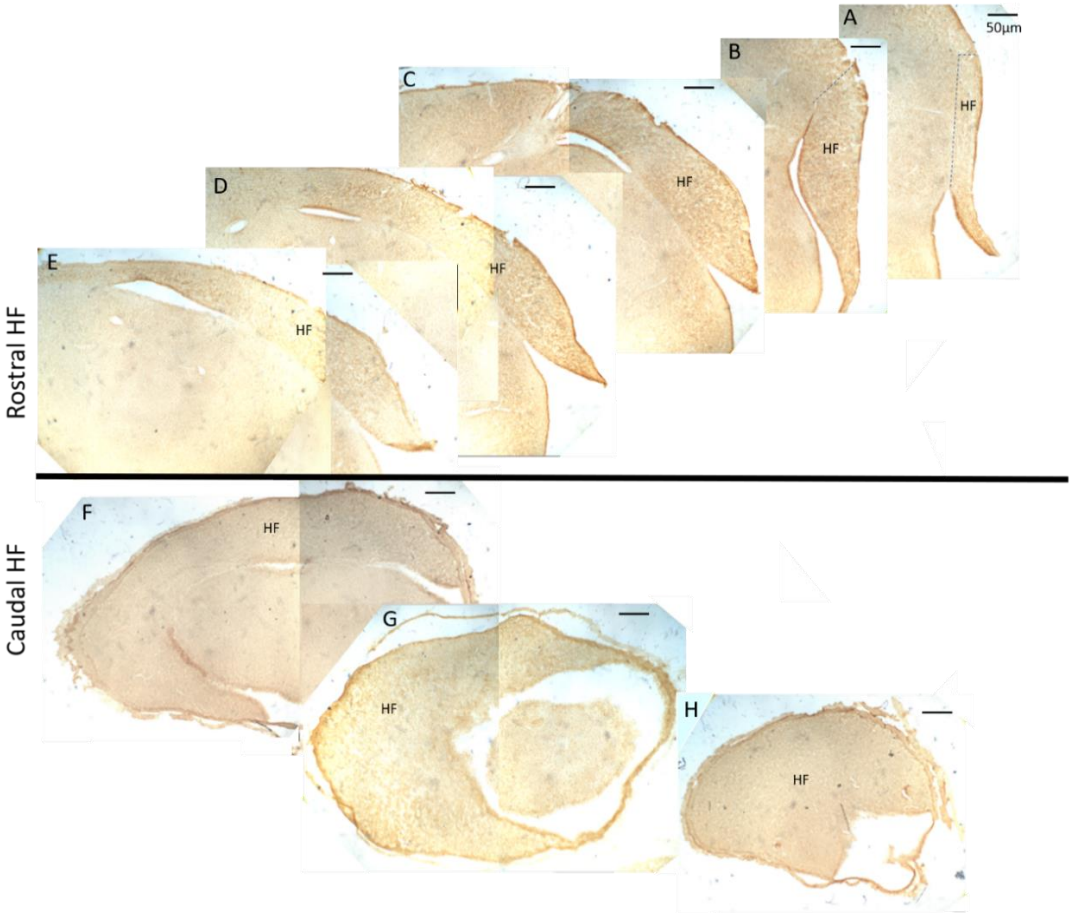

Figure 4.1. Representative 50 µm-thick doublecortin-stained coronal sections from across the longitudinal axis of the adult hen hippocampal formation (HF). Sections A–E contain the rostral HF and are 1600 µm apart; sections F–H contain the caudal HF and are 400 µm apart. Scale bar is 50 µm wide.

Image analysis was performed with Stereo Investigator software (version 2018.1.1, MBF Bioscience, USA). Hippocampal borders were outlined at 2.5X magnification (0.07 numerical aperture) on every analysed slice according to the chick stereotaxic atlas (Puelles, 2007). Because of the complex structure of the avian HF, we divided the whole structure in two major components: i) the rostral hippocampus (interaural 5.68/0.50) and ii) the caudal hippocampus (interaural 0.50/-0.50). Cell counting was performed at 100X magnification (0.65 numerical aperture). Stereological parameters were set to an optical fractionator grid of 120 x 120 µm for the rostral HF and 240 x 240 µm for the caudal HF, a counting frame of 50x50 µm for both regions and a mounted thickness of 20 µm.

Although no granule cells are present in the avian HF, different types of DCX^+ cells have been previously described in the avian brain literature (Boseret *et al.*, 2007; Melleu *et al.*, 2016) and can be divided into two groups according to neuronal morphology: I) multipolar neurons and II) bipolar (fusiform) cells. In line with Boseret *et al.*, we assume that the fusiform neurons are younger and still migrating, while the multipolar neurons are more mature and settling. Multipolar cells were defined as medium-large sized cells, with a round or polygonal/angular cell body shape and process branching from it in three or more directions. Golgi analysis of the chick HF (Tömböl *et al.*, 2000) suggests that this group includes a high proportion of multipolar projection neurons, but may also incorporate pyramidal and multipolar local circuit neurons. Bipolar/fusiform cells were defined as medium-small sized cells with elliptical or oval cell body shape and process branching from it in two or fewer directions. Examples of stained cells with these morphologies are displayed in Figure 4.2. Cells of these two types lying inside the optical fractionator frame or bisected by its green lines were counted, according to the Optical Fractionator method.

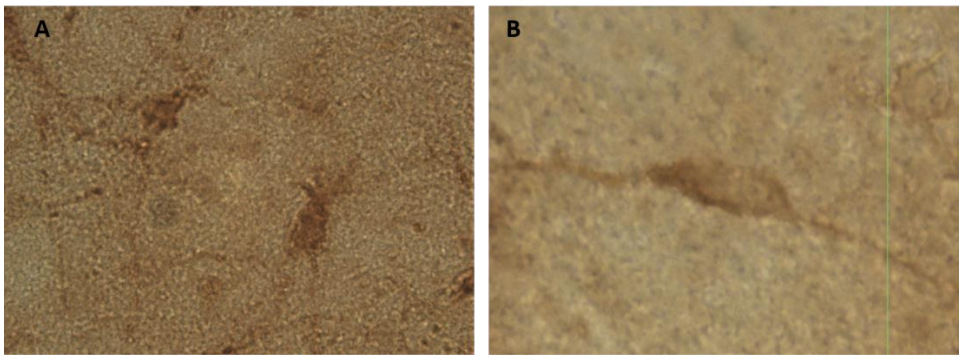

Figure 4.2. Examples of doublecortin-labelled cells with a) multipolar and b) bipolar/fusiform morphologies. Images (not to scale) were captured at 100x magnification.

Counts were exported to MS Excel from Stereo Investigator and used to manually calculate

densities of each cell type. Specifically, the number of counted cells of each type was divided by the area of the counting frame (2500µm), multiplied by both the number of counting sites sampled in that brain and the section thickness (50µm), to produce a density per volume measure for the sampled tissue. Values were transformed to densities per cubic millimetre by multiplying by 10^9. The rostral and caudal hippocampus were treated separately.

4.3.9 Molecular biology

Homogenised tissue from the rostral and caudal HF of the remaining forebrain hemisphere from each hen was used to quantify mRNA for *DCX* and housekeeping gene *LBR*, with RNA extracted and reverse transcribed into cDNA according to the methods described in chapter 2 (section 2.3.6). Quantitative PCR was conducted as in section 2.3.8, using the same gene-specific primers as in previous studies (Table 2.1) and utilising gene-specific template produced previously (section 2.3.7) for standard curves. In this case, the 48 HF samples could be reverse transcribed in a single batch and processed using a single qPCR assay per gene. Measured molar quantities of *DCX* and *LBR* mRNA were log(10)-transformed for statistical analysis.

4.3.10 Statistical analysis

When analysing radiographs from the final time point (T11) after tissue collection, it was necessary to move three sampled birds from the minimal to the severe KBF group for the purpose of statistical analysis. Final sample sizes for between-group analyses were thus 9 minimal KBF and 15 severe KBF hens. Descriptive statistics are displayed as mean (M) ± standard deviation (SD), and all analyses were run in IBM SPSS Statistics (v24). To compare activity of the minimal and severe KBF groups at time point 1 (before most fractures occurred) and time point 11, a linear mixed model (LMM) was conducted for the mean aviary transitions made during the 6 days preceding each. Experimental pen was included as a random factor, with time point as a repeated fixed factor and final KBF severity group as a between-subject fixed factor. To assess the relationship between KBF severity and AHN, separate LMMs were conducted for raw densities of DCX-expressing multipolar and bipolar cells. In all models, staining batch and experimental pen were included as random factors, whilst HF subregion (rostral/caudal) was included as a repeated fixed factor. When exploring group differences, fracture status at the final time point (minimal/severe KBF) was included as a between-subject fixed factor, and in an interaction term with HF subregion. To assess within-individual relationships, KBF severity score at the final time point (T11) and the mean number of transitions made between aviary zones over the preceding 6 days were entered as covariates in separate models, each of which included their interaction with HF subregion. To determine

which variable best predicted cell densities, a model including both covariates (and their interactions with subregion) was also run. Several analyses were conducted in order to explore the influence of the timescale of KBF acquisition on AHN at the end of life. Firstly, the time point at which a hen suffered its first KBF was included as a covariate in LMMs for the two cell types, again alongside staining batch and pen as random factors, HF subregion as a fixed factor and the interaction between time point and subregion. Secondly, to determine at which time point fracture severity best predicted DCX^+ cell densities, and if severity at any other times added explanatory power, LMMs including severity score at each time as a covariate were conducted in a stepwise forward manner. Lastly, the changes in KBF severity compared to the previous recorded score at each time point were included as covariates in LMMs, according to the same stepwise procedure. These analyses were conducted separately for DCX^+ multipolar and bipolar densities. All figures display cell densities normalised (z-scored) within their staining batch.

To analyse expression of *DCX* mRNA, LMMs were conducted with HF subregion as a repeated fixed factor, experimental pen as a random factor, and *LBR* expression as a covariate. Separate models included either KBF severity group as a between-subject fixed factor, or final KBF severity score as a covariate, and in an interaction term with HF subregion. Finally, the concordance between molecular measurements and cell counts was assessed in terms of the relationship between DCX^+ cell densities and unstandardized residual expression of *DCX* mRNA, after controlling for expression of *LBR* in a simple linear regression. LMMs were therefore conducted with measures of DCX^+ cell densities (multipolar, bipolar and combined) as the dependent variables and residual *DCX* mRNA expression as a covariate. Each included staining batch and pen as random factors, and HF subregion and KBF severity group as repeated fixed factors.

4.4 Results

Subsequent to readjusting sample groups based on KBF severity in radiographs taken at the final time point, hens in the minimal KBF group (n = 9) had a mean fracture severity score of 3.5 (± 0.87 SD), with individual scores ranging from 1.9 - 4.8. In contrast, hens in the severe KBF group (n = 15) had a mean final fracture severity of 8.5 (± 0.85 SD) and a range of 7.2 - 10.0.

4.4.1 Changes in activity levels

The mean number of aviary transitions made during the 6 days preceding the first and last study time points (T1 and T11) was compared for hens who had developed minimal versus severe KBFs by the latter time point (see Figure 4.1). Due to some loss of signal, transitions at both of these time points were successfully recorded for 6 minimal and 13 severe KBF hens. At time point 1, all hens in the minimal KBF group had severity scores of 0, which was also the case for all but two individuals in the severe KBF group (scoring 3.0 and 4.0). The KBF groups did not differ in their overall vertical transitions ($F_{1,22.2} = 0.010$, $p = 0.921$), whilst the number of transitions made by all hens decreased between time point 1 (M = 75.0 ± 40.1 SD) and time point 11 (M = 35.5 ± 29.7 SD, $F_{1,21.5} = 14.35$, $p = 0.001$). An interaction existed between study time point and KBF severity group ($F_{1,21.5} = 5.996$, $p = 0.023$). Whereas there was no difference in the number of transitions made by minimal KBF birds at T1 (M = 60.40 ± 24.5 SD) and T11 (M = 48.0 ± 10.8 SD; $p = 0.422$), severe KBF birds made fewer transitions at T11 (M = 29.7 ± 34.1 SD) than they had at T1 (M = 82.8 ± 45.1 SD; $p < 0.001$). Minimal and severe KBF birds did not differ from each other in the absolute number of transitions made at either T1 ($p = 0.209$) or T11 ($p = 0.169$).

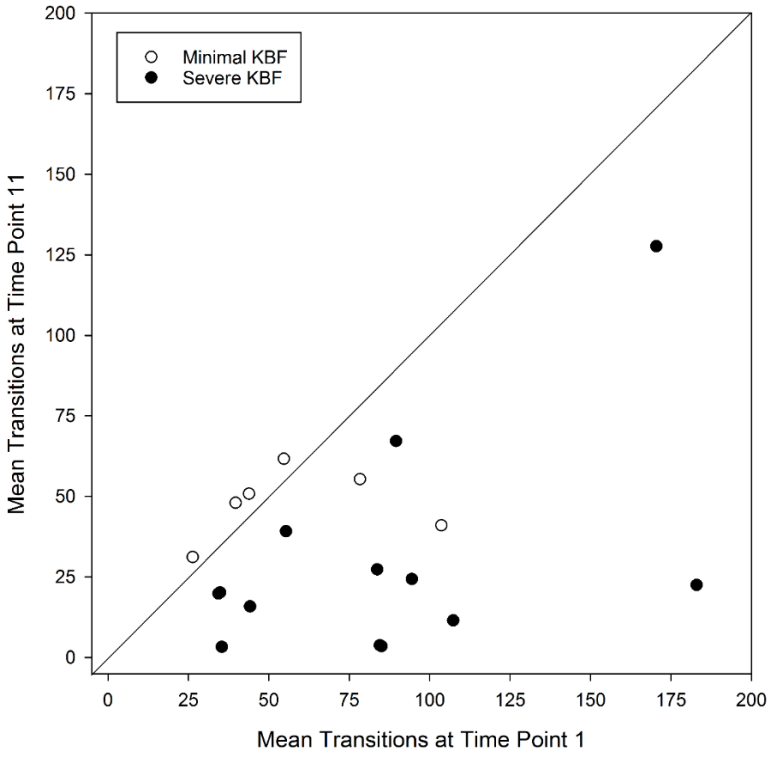

Figure 4.3. The mean number of vertical aviary transitions made by hens which had developed minimal versus severe keel bone fractures (KBFs) by time point 11, made during the 6 days preceding the first (T1) and final (T11) radiograph time points. Line of equality represents equal activity at both time points. Some data points are overlapping.

4.4.2 Adult hippocampal neurogenesis & KBF severity group

Hens with severe KBFs 3-4 weeks before tissue sampling had fewer DCX^+ multipolar cells than counterparts with minimal fractures in the HF as a whole ($F_{1,14.5} = 22.56$, $p < 0.001$), and there was no effect of HF subregion ($F_{1,20.2} = 2.93$, $p = 0.102$). There was an interaction between subregion and fracture status ($F_{1,20.2} = 10.52$, $p = 0.004$, Figure 4.2a), whereby hens with minimal fractures had more DCX^+ multipolar neurons than hens with severe fractures in both the rostral ($p = .011$) and caudal ($p < 0.001$) HF, but the effect was stronger in the caudal HF. Hens with minimal fractures alone had a higher density of stained neurons in the caudal HF than the rostral region ($p = 0.001$), with no subregional difference in hens with severe fractures ($p = 0.294$).

Hens suffering severe KBFs also had fewer DCX^+ bipolar cells throughout the HF ($F_{1,17.5} = 12.48$, $p = 0.002$). Cell counts did not differ between the rostral and caudal regions ($F_{1,\ 21.9} = 3.45$, $p = 0.077$), nor did subregion interact with fracture status ($F_{1,21.9} = 0.069$, $p = 0.796$, Figure 4.2b).

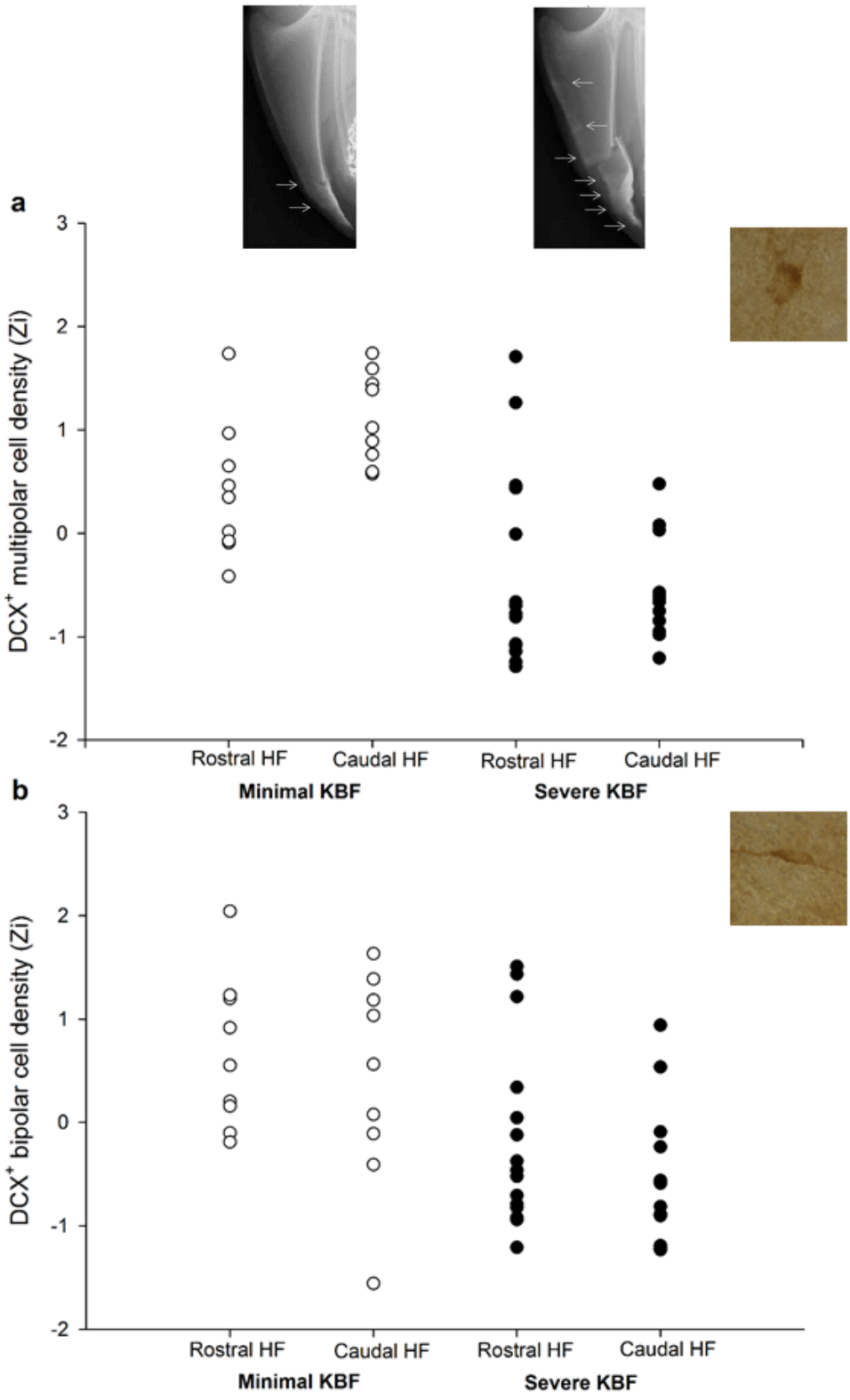

Figure 4.4. Densities of a) multipolar and b) bipolar DCX-stained cells in the rostral and caudal HF subregions of hens with minimal versus severe keel bone fractures (KBFs), normalised (Z_i) for staining batch. Example radiographs reflect the mean severity scores of the minimal and severe KBF groups: 3.5 and 8.5 respectively.

4.4.3 Adult hippocampal neurogenesis & KBF severity score

Final KBF severity (3-4 weeks prior to tissue collection) negatively predicted DCX^+ multipolar cell density across individual birds ($F_{1,16.8} = 35.98$, $p < 0.001$). With severity accounted for, the effect of HF subregion reached significance, with more DCX^+ multipolar neurons present in the caudal HF ($F_{1,19.8} = 9.17$, $p = 0.007$). KBF severity interacted with subregion, ($F_{1,20.5} = 7.99$, $p = 0.010$), reflecting a steeper relationship between severity score and multipolar cell density in the caudal HF (B = -134.37, SEM = 19.22, $F_{1,18.1} = 48.87$, $p < 0.001$) than in the rostral region (B = -89.42, SEM = 23.68, $F_{1,15.4} = 14.30$, $p = 0.001$, Figure 4.3a).

Final fracture severity negatively predicted densities of DCX^+ bipolar neurons ($F_{1,17.4} = 11.96$, $p = 0.003$, Figure 4.3b). There was no main effect of HF subregion ($F_{1,21.7} = 0.460$, $p = 0.505$), nor was there an interaction between severity score and subregion ($F_{1,22.3} = 0.003$, $p = 0.960$).

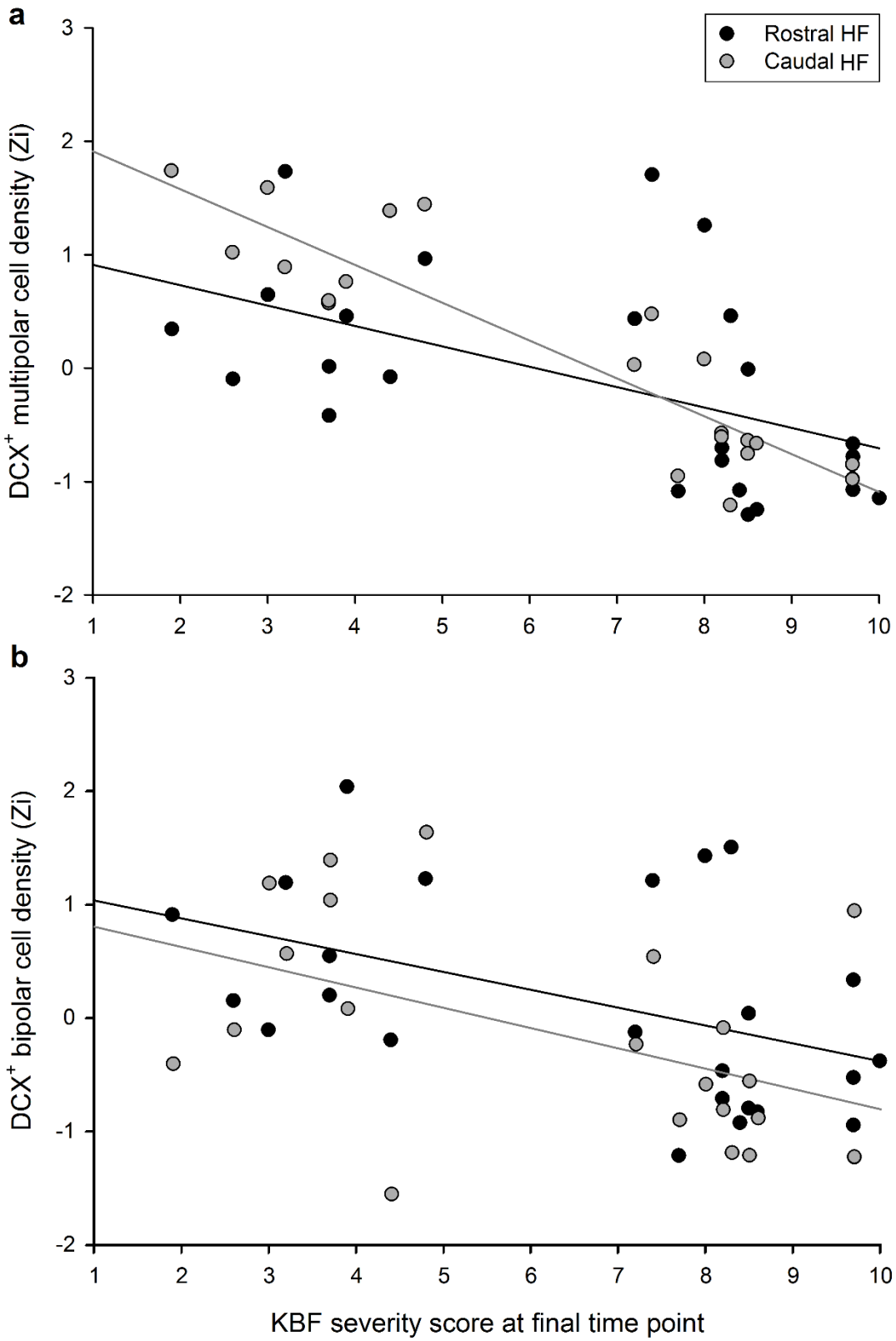

Figure 4.5. Relationship between keel bone fracture (KBF) score at the final time point (ranging from 0 = no KBF to 10 = extremely severe) and density of DCX-positive (a) multipolar and (b) bipolar cells in the rostral and caudal HF subregions, normalised (Z_i) for staining batch. Simple linear regression lines are plotted for cell densities in the rostral (black) and caudal (grey) HF subregions.

4.4.4 AHN & activity

Mean number of daily aviary transitions at the final time point (3-4 weeks before tissue sampling) did not predict multipolar DCX^+ cell densities ($F_{1,14.2} = 0.271$, $p = 0.611$) and did not interact with HF subregion ($F_{1,15.0} = 0.633$, $p = 0.439$). Similarly, mean transitions did not co-vary with density of DCX^+ bipolar cells ($F_{1,14.3} = 0.032$, $p = 0.860$; transitions*subregion: $F_{1,15.9} = 0.135$, $p = 0.718$). Furthermore, including transitions as a covariate in LMMs along with KBF severity score did not remove the effect of KBF severity, which continued to negatively predict densities of DCX^+ multipolar ($F_{1,11.2} = 22.89$, $p = 0.001$) and bipolar ($F_{1,11.5} = 21.62$, $p = 0.001$) cells in the HF.

4.4.5 Timescale of KBF acquisition

For hens that had developed severe KBFs by the final time point (T11), the median time point for acquisition of the first fracture was T3, compared to one time point later (T4) for hens with only minimal damage by the end of the study. Though time point at which a KBF was first acquired did not co-vary with AHN over the whole HF ($F_{1,16.2} = 1.87$, $p = 0.190$), it interacted with subregion to predict DCX^+ multipolar cell density ($F_{1,20.0} = 17.63$, $p < 0.001$). There was a trend for hens that acquired KBFs earlier and thus had suffered them for longer to have fewer new multipolar neurons at the caudal pole (B = 118.15, SEM = 57.51, $F_{1,16.1} = 4.22$, $p = 0.057$), with no relationship in the rostral HF (B = 9.81, SEM = 51.79, $F_{1,19.2} = 0.036$, $p = 0.852$) (Figure 4.4a). A similar effect was observed for bipolar cells (time point $F_{1,20.0} = 0.267$, $p = 0.611$, time point*subregion: $F_{1,21.8} = 7.33$, $p = 0.013$, Figure 4.4b).

The time point at which a hen acquired their first KBF was moderately negatively correlated with their fracture severity at the final time point (T11), $r(22) = -0.561$, $p = 0.004$. However, time point of first damage continued to interact with HF subregion to predict caudal DCX^+ multipolar density when included in the same LMM as KBF severity at T11 (first damage time point*subregion: $F_{1,18.7} = 7.58$, $p = 0.013$; first damage timepoint: $F_{1,13.8} = 0.649$, $p = 0.434$; severity T11: $F_{1,12.0} = 29.24$, $p < 0.001$; severity T11* subregion: $F_{1,18.4} = 2.189$, $p = 0.156$). This was also the case for DCX+ bipolar cell density (first damage time point*subregion: $F_{1,20.0} = 11.10$, $p = 0.003$).

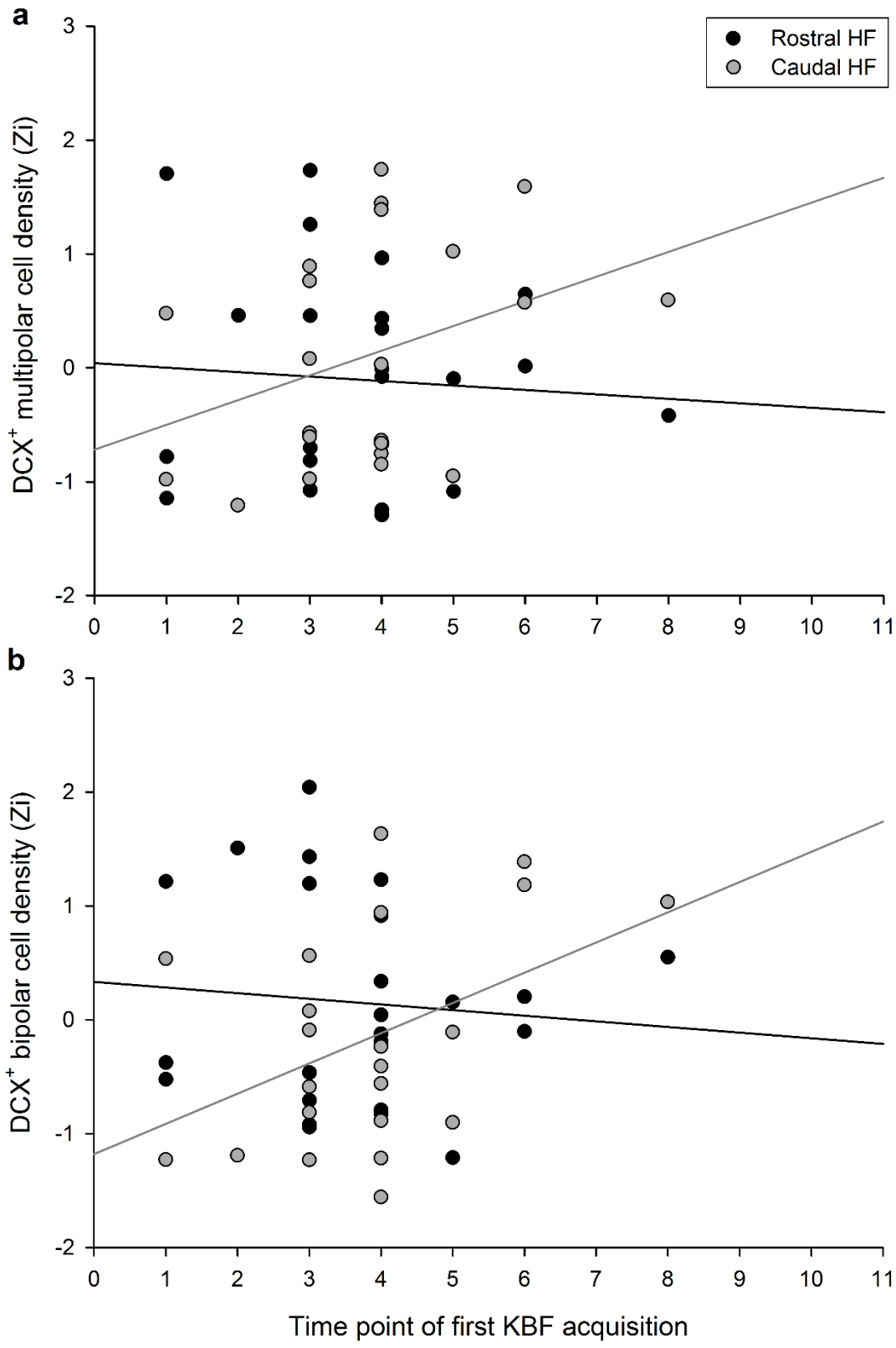

Figure 4.6. Relationship between the time point at which individual hens first developed a keel bone fracture (KBF) and their density of DCX-positive (a) multipolar and (b) bipolar cells in the rostral and caudal HF, normalised (Z_i) for staining batch. Simple linear regression lines are plotted for cell densities in the rostral (black) and caudal (grey) HF subregions.

LMMs conducted in a stepwise forward manner, with KBF severity at each time point as covariates, confirmed that severity at the final time point (T11) was the best predictor of DCX^+ multipolar densities over the whole HF at end of life (results reported in section 4.4.3), with severity at no other time point adding significant explanatory power. Although KBF severity at T11 was the best predictor, when analysing each time point separately, end of life densities of DCX^+ multipolar cells were predicted by KBF severity scores from time point 4 onwards (T4 severity: $F_{1,17.1} = 8.11$, $p = 0.011$; $p \leq 0.001$ for severity at all subsequent time points). DCX^+ bipolar densities were best predicted by KBF severity at time point 10 ($F_{1,18.1} = 14.12$, $p = 0.001$), and other time points did not add further explanatory power to the model. There was a trend towards bipolar densities being predicted by severity at time point 4, ($F_{1,18.9} = 3.88$, $p = 0.064$), which remained significant for time point 5 onwards ($p \leq 0.012$).

To explore the integration window over which AHN assimilates changes in the experience of pain/stress, the same stepwise LMM procedure was conducted for the difference in KBF severity at each time point compared to the previous recorded score. Change in KBF severity between time points 4 & 5 ($F_{1,18.9} = 18.61$, $p < 0.001$), 3 & 4 ($F_{1,15.2} = 6.80$, $p = 0.020$) and 9 & 10 ($F_{1,17.1} = 5.58$, $p = 0.030$) negatively predicted DCX^+ multipolar cell densities at end of life (Figure 4.5a & b). Changes in severity between time points 4 & 5 ($F_{1,19.1} = 13.57$, $p = 0.002$) and 3 & 4 ($F_{1,14.7} = 7.39$, $p = 0.016$) similarly predicted DCX^+ bipolar cell densities, though severity difference between time points 9 & 10 did not ($F_{1,19.4} = 0.322$, $p = 0.577$, Figure 4.5c & d).

The predictive power of change in severity between these particular time points did not arise from their contribution to KBF severity at the final time point, as these variables were not correlated (T3 to 4: $r(22) = 0.187$, $p = 0.381$; T4 to 5: $r(22) = 0.170$, $p = 0.426$: T9 to 10: $r(22) = 0.339$, $p = 0.105$).

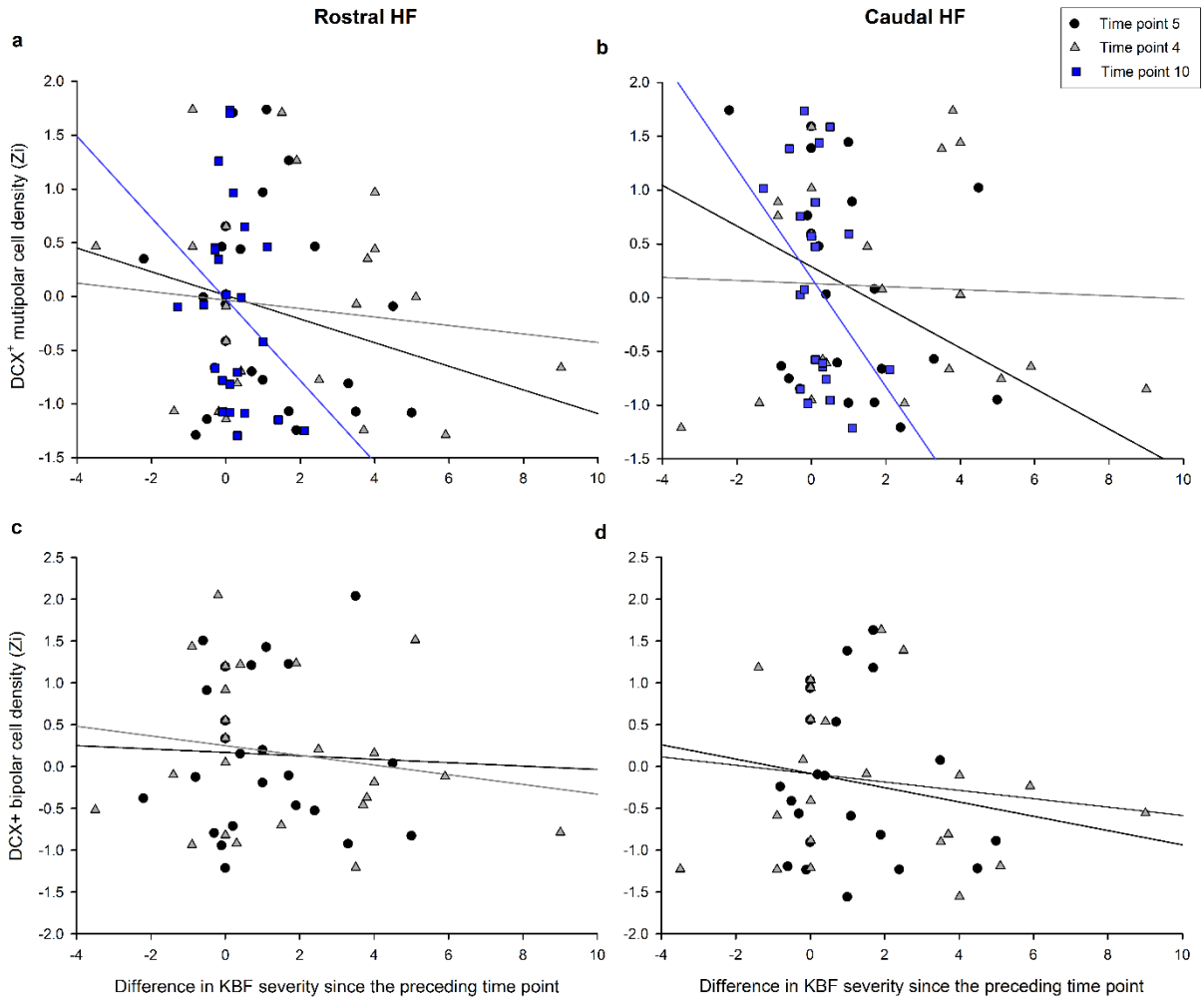

Figure 4.7. The relationship between the difference in keel bone fracture (KBF) severity score since the preceding time point and densities of DCX+ (a) rostral multipolar, (b) caudal multipolar, (c) rostral bipolar and (d) caudal bipolar cells (Z_i), for time points wherein this change in severity predicted cell densities at end of life. Simple linear regression lines are plotted for time points 5 (black), 4 (grey) and 10 (blue).

4.4.6 Quantification of DCX mRNA

As expected, expression of housekeeping gene *LBR* significantly predicted *DCX* mRNA expression ($F_{1,38.2} = 668.45$, $p < 0.001$), while *DCX* mRNA expression was higher in the caudal HF than in the rostral subregion ($F_{1,21.2} = 7.29$, $p = 0.013$). Expression of *DCX* mRNA did not significantly differ between KBF severity groups ($F_{1,21.1} = 0.377$, $p = 0.546$), but there was a trend towards an interaction with HF subregion ($F_{1,21.8} = 3.59$, $p = 0.072$). Minimal KBF hens have more *DCX* mRNA in the caudal HF than the rostral subregion ($p = 0.009$), whereas there was no subregional difference for the severe KBF group ($p = 0.527$). Expression of *DCX* mRNA did not significantly differ between hens with minimal and severe KBFs in either the rostral ($p = 0.621$) or caudal ($p = 0.132$) subregion (Figure 4.6). When included as a covariate, KBF

severity score at the final time point (T11) did not predict *DCX* mRNA expression ($F_{1,20.6}$ = 1.11, p = 0.305) and did not interact with HF subregion ($F_{1,21.0}$ = 2.48, p = 0.131).

Expression of *DCX* mRNA within a HF tissue sample did not significantly predict DCX^+ multipolar ($F_{1,30.7}$ = 1.99, p = 0.168), bipolar ($F_{1,29.7}$ = 0.66, p = 0.423), or combined (multipolar + bipolar) cell densities ($F_{1,31.6}$ = 0.01, p = 0.945), quantified in serial hippocampal sections from the other forebrain hemisphere of the same hens.

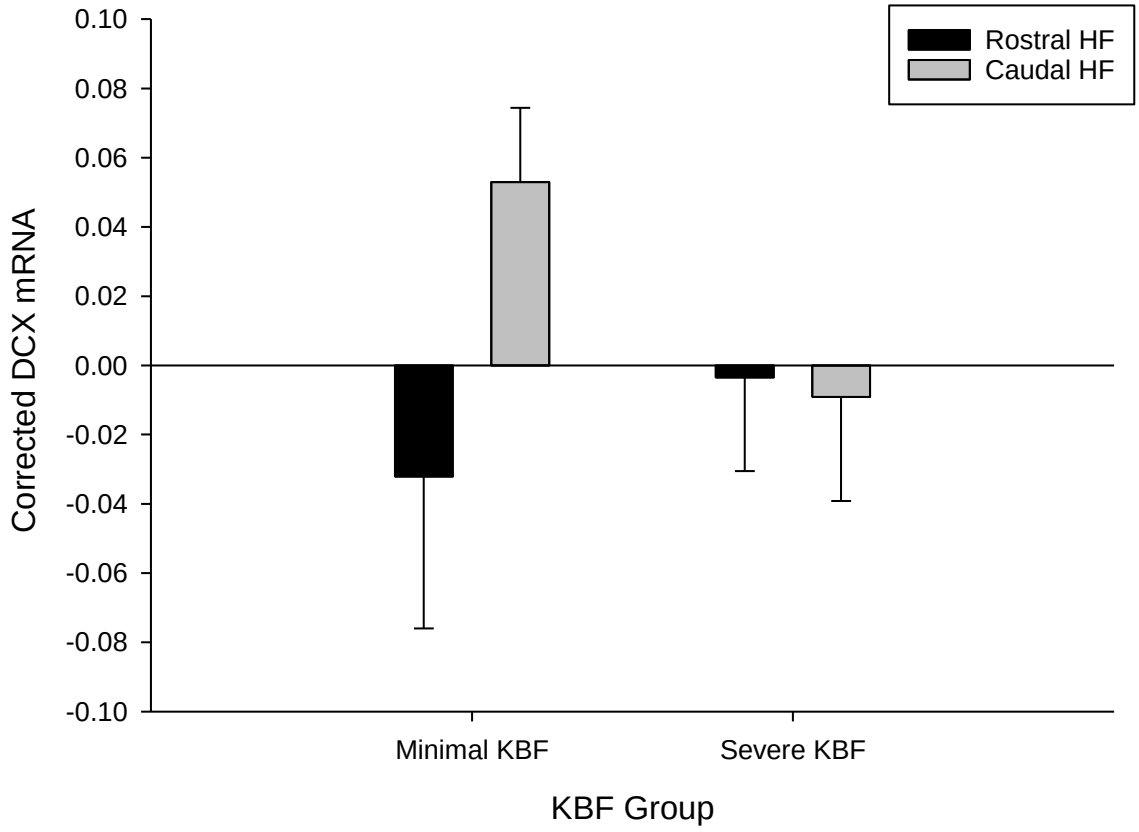

Figure 4.8. Residual expression of doublecortin (*DCX*) mRNA in the rostral and caudal HF subregions of hens with minimal versus severe keel bone fractures (KBFs) at the final study time point, after controlling for expression of *LBR*.

4.5 Discussion

4.5.1 Implications for laying hen welfare

Hens with severe KBFs 3-4 weeks before tissue sampling had lower densities of DCX^+ multipolar and bipolar neurons in the HF when compared to birds with minimal KBFs, while the magnitude of keel bone injury an individual had sustained by this time linearly predicted their number of new-born differentiating cells. Such downregulated AHN occurs as part of a depression-like physiological state, arising from chronic exposure to stress across numerous

paradigms in mammalian (Gould *et al.*, 1998; Gould and Tanapat, 1999; O'Leary and Cryan, 2014) and avian (Barnea and Nottebohm, 1994; LaDage *et al.*, 2010; Robertson *et al.*, 2017; Gualtieri *et al.*, 2019) species. As previous work provided behavioural evidence of pain through a place preference for the location of analgesic administration in hens with KBFs (Nasr *et al.*, 2013), altered HF morphology in terms of reduced AHN in the present study further suggests that the accumulation of fractures is sufficient to lead to long-term stress in affected birds. In mice, models of chronic neuropathic pain severe enough to suppress AHN are also associated with anxiety- and depressive-like behaviour (Mutso *et al.*, 2012; Dimitrov *et al.*, 2014; Zheng *et al.*, 2017). These neural and affective changes are observed to persist beyond reversal of the injury (Dimitrov *et al.*, 2014), indicating that they are not an immediate result of nociceptive stimulation. Given that the hens in the present study showed a similar reduction in AHN following chronic pain, it can be inferred that an accompanying depressive-like state was likely induced.

In mice, a more severe model of neuropathic injury led to a greater quantitative reduction in DCX^+ hippocampal cells (Dimitrov *et al.*, 2014), while in the present study the magnitude of keel bone damage present 3-4 weeks before end of life linearly predicted the suppression of neuronal differentiation in the laying hen HF. The interpretation that these immature cell numbers in turn reflect the relative levels of stress experienced by individual hens is supported by the previous observation of lower levels of AHN in rats exposed to neuropathic pain combined with another source of stress (chronic immobilisation), compared to those exposed to either of these stressors alone (Romero-Grimaldi *et al.*, 2015). Adult-born neurons in the temporal mouse HF act to inhibit those mature neurons in the same region that respond preferentially to anxiogenic conditions (Anacker *et al.*, 2018), and factors that stimulate and suppress AHN (enrichment and aging, respectively) influence the degree of inhibition evoked by these young neurons accordingly (Drew *et al.*, 2016). Though currently unexplored, if adult-born cells in the chicken HF are similarly involved in inhibition of stress-responsive mature neurons, this linear relationship may relate to a cumulative loss of this stress resilience with increasing magnitude and/or duration of pain.

Though individual differences in activity levels masked differences between minimal and severe KBF hens in the absolute number of vertical aviary transitions made at the final time point, hens that had developed severe KBFs by this time made fewer transitions than they had at the first time point, before most individuals had suffered any damage. In contrast, activity levels of hens that developed only minimal KBF did not change between the first and final time points. This indicates that birds with severe KBFs, as selected for the present sample, show a

corresponding reduction in activity. The assumption that it is painful for them to move is consistent with reported analgesic-driven reductions in latency for hens with KBFs to descend from raised perches (Nasr *et al.*, 2012b). On the other hand, lower AHN in hens with severe fractures could not be explained by their recorded activity 3-4 weeks before tissue sampling, and thus cannot be attributed to a consequence of varying levels of activity or concurrent spatial cognitive processing at this time. Our findings thus support the inference that hens with KBFs experience a corresponding negative affective state beyond an acute sensory pain response. Since this is likely to influence overall quality of life for laying hens in commercial systems, management steps to reduce or delay acquisition of KBFs are likely to have a notable impact on welfare.

4.5.2 Timescale for integration of experience

Quantification of PCNA and Ki-67$^+$ progenitor cells in the rodent hippocampus demonstrates that chronic pain lowers rates of proliferation, which continue to be downregulated for at least 35 days after the initial injury, or three weeks after it is reversed (Dimitrov *et al.*, 2014; Romero-Grimaldi *et al.*, 2015). Proliferation levels are reflected proportionately in subsequent net survival, even when the modulatory stimulus is discontinued during later development (Fabel *et al.*, 2009). Stress also influences how many immature neurons developing at the time of this experience survive to be recruited for terminal differentiation and incorporation into the neural circuity (Lee *et al.*, 2006; Lehmann *et al.*, 2013; Castilla-Ortega *et al.*, 2014). Studies in rodents have not yet measured the comparative survival rate of BrdU$^+$ cells that were labelled before the induction of chronic pain. However, while the results obtained in the present study were qualitatively similar for multipolar and bipolar/fusiform DCX$^+$ cells, assumed to be respectively more mature versus younger and still migrating (Boseret *et al.*, 2007; Melleu *et al.*, 2016), relationships with KBF severity were stronger for the multipolar cells. This suggests that the chronic stress arising from severe KBFs indeed influences the survival of immature neurons present at the time it is experienced, with cells at later phases of neuronal development being particularly sensitive. Studies in rodents that have distinguished between subtypes of DCX$^+$ cells according to their morphology have similarly reported greater reductions in numbers of relatively more mature differentiating neurons during exposure to chronic social (Van Bokhoven *et al.*, 2011) and intermittent restraint (Castilla-Ortega *et al.*, 2014) stress regimes. As such, the observed relationships between KBF severity and DCX$^+$ cell densities are likely to result from a combination of downregulated proliferation (occurring 3-30 days previously), and reduced survival of those neuroblasts recently undergoing later differentiation.

Previous research has indicated that it is common for hens to sustain multiple fractures to the keel at different points throughout the laying cycle, with a mean of 3.1 (± 1.8 SD) KBFs per hen observed at the end of the laying period in a similar study (Baur *et al.*, 2020). Multiple fractures were also observed in the present study, but due to variability in dimensions, location, healing status, etc., quantified KBF severity scores collated all fractures present (both existing and new) (Rufener *et al.*, 2018b), and thus tended to increase incrementally after damage was first sustained. Though it is not known for how long a KBF continues to be a source of pain after acquisition, hens that developed a conditioned place preference for the location of analgesic administration in a previous study had had KBFs for at least three weeks prior to the point of testing (Nasr *et al.*, 2013), whilst hard callus formation takes four to six weeks to occur (Richards *et al.*, 2011). Radiographs in the present study were taken four to five weeks apart, leading to two possible interpretations of analyses relating the timescale of KBF severity to AHN (i.e. the collective result of modulated proliferation and survival). The first is based upon the assumption that, after this period of time, previous fractures have healed to the extent that they are no longer painful or stressful, and thus would only have exerted a suppressive effect on AHN during their recorded time point. Therefore, hens with more recent (still healing) fractures should be more affected, which is supported by the finding that severity of most recent KBFs (radiographed 3-4 weeks prior) were the best predictor of AHN. In this case, where hens with older fractures also show corresponding reductions in AHN, the window over which AHN integrates experience must span this length of time. Our results indicate that AHN at end of life is predicted by KBF severity from around time point 4 onwards, meaning AHN would thus integrate experience from around 34 weeks previously.

The alternative interpretation is based on the assumption that healed breaks remain somewhat painful, and therefore stressful, over a hen's lifetime. Indeed, existing KBFs may often be repeatedly aggravated during normal hen behaviour, such as movement to food or water, flight and perching (Riber *et al.*, 2018). If this is the case, through integrating older and newer fractures, severity score at the final time point in itself reflects the cumulative pain or discomfort experienced. This means it is not possible to determine the time scale over which AHN integrates painful experience, as that occurring in the past persists until the end of life. As such, the contribution of recent versus longer-term pain or stress to the relationship between AHN and KBF severity measured at the latest point (3-4 weeks previously) cannot be distinguished. However, in the caudal HF subregion, a relationship existed between AHN and the duration of time since fractures were first incurred. Moreover, this variable predicted caudal AHN independently of severity at the final time point. Furthermore, changes in KBF severity score

since the preceding time point occurring as early as ~37 weeks previously also predicted AHN over the whole HF, and were not correlated with final fracture severity. These relationships were not as strong as those with end of life KBF severity, suggesting that AHN may integrate temporally distant experience on a more subtle level, alongside that which is most recent or cumulative. Before future studies definitively elucidate the time scale over which KBFs cause pain, it is not possible to know which assumption is correct, meaning the results from the present study cannot determine the window of time over which AHN integrates negative experience.

4.5.3 Homologies with mammalian AHN

In the present study, KBFs were associated with suppressed AHN over the whole laying hen HF, rather than being restricted to the putatively more stress-sensitive caudal region (Smulders, 2017). In rodents, some models of chronic stress have a temporal-specific influence on AHN, whereas others result in suppression across the whole dentate gyrus (see O'Leary and Cryan (2014) for a review). Whether the effect is regionally-localised may depend on the nature of the stressor or paradigm employed, and mouse models of chronic pain have produced qualitatively similar reductions in AHN in both HF subregions (Dimitrov *et al.*, 2014; Zheng *et al.*, 2017). Therefore, whereas a sequence of mild, albeit unpredictable stressors (as in Gualtieri *et al.*, 2019) appears to produce a caudal-specific suppression of laying hen AHN, sources of chronic pain such as KBFs may conceivably present stressors of greater magnitude, which affect neurogenesis across more of the HF. That being said, for multipolar DCX^+ neurons, the interaction between individual KBF scores and HF subregion demonstrates that the suppressive effect of these fractures is stronger at the caudal pole. Moreover, the caudal HF appears more sensitive to temporal aspects of KBF acquisition, indicated by an interaction between HF subregion and the duration of time since fractures were first incurred. While DCX^+ multipolar neuron densities in the rostral HF were not related to the time since KBFs first occurred, a tendency for a negative relationship existed within the caudal HF. Interestingly, the temporal mouse hippocampus, thought to be homologous to the caudal avian HF (Smulders, 2017), appears to be preferentially involved in the affective component of pain. Ablating AHN in this region attenuates the anxiolytic effects of environmental enrichment and exercise on behaviour in the open field and EPM during chronic inflammatory pain (Zheng *et al.*, 2017).

In contrast to fracture severity, the number of transitions hens made between aviary zones 3-4 weeks before tissue sampling was not associated with AHN when employed as a proxy for activity. Such a relationship might have been predicted based on rodent studies, wherein time spent voluntarily running in a wheel correlates positively with AHN (Allen *et al.*, 2001; Rhodes

et al., 2003). However, there is less evidence for a conserved relationship between exercise and AHN in birds, and flight exercise was previously found not to be associated with DCX-expression in starlings (Hall *et al.*, 2014). Infrared tracking in the present study also recorded only vertical activity within the aviary, meaning individual differences in activity within a single horizontal zone or tier were not accounted for. Further exploration into the association between exercise and AHN in avian species may therefore be required in future. It would be worthwhile to consider more complex assessments of hen movement, that account for consistency and changes in patterns over multiple days and longer periods of a hens' life (as in Rufener *et al.*, 2018c).

4.5.4 Molecular measurement of DCX expression

Molecular measurement of *DCX* mRNA mirrored the observed subregional difference in hippocampal DCX^+ stained cell density, but was less sensitive in reflecting the impact of varying KBF severity on neuronal differentiation. In theory, a greater density of differentiating neurons should be reflected by more synthesis of the DCX protein, requiring an increased concentration of *DCX* mRNA transcripts. However, a within-bird comparison indicated that *DCX* mRNA expression levels did not predict cell counts in the equivalent HF subregion of the alternate hemisphere. In mice, damage restricted to a single paw in mice still leads to a bilateral suppression of AHN (Mutso *et al.*, 2012), suggesting that if regulatory mechanisms are indeed conserved between mammals and birds, the observed discrepancy is unlikely to be due to a lateralized sensitivity to chronic pain. However, explorations in homing pigeons highlight notable functional lateralization in the avian HF (Bingman *et al.*, 2006; Jonckers *et al.*, 2015), which may exceed that in rats and mice. The contribution of hemispheric factors can therefore not be entirely ruled out. Additionally, studies have highlighted several biological mechanisms that lead to the decoupling of mRNA and protein levels (Fortelny *et al.*, 2017). Among other factors, translation rates are influenced by the specific mRNA sequence and modulated by the local availability of resources for protein biosynthesis (Liu *et al.*, 2016). Individual cells also vary in their overall transcription of particular genes, and these variations correlate strongly with cell volumes (Padovan-Merhar *et al.*, 2015). Additional to cell number, soma size and the extent of neuronal processes may therefore influence concentrations of mRNA in homogenized tissue. Moreover, weakly labeled DCX-immunoreactive structures have been observed in various di- and mes- encephalic nuclei of the adult canary brain and, as new neurons are not incorporated in these regions, are assumed not be associated with AHN (Boseret *et al.*, 2007; Balthazart *et al.*, 2008). Background expression of DCX mRNA and protein has also been noted in non-neurogenic subdivisions of the mouse hippocampus, with levels which are unresponsive

to running exercise (Kremer *et al.*, 2013). This observation is perhaps attributable to expression of DCX in mature neurons during processes of cellular remodeling, such as reorganization of the dendritic arbor (Nacher *et al.*, 2001; Brown *et al.*, 2003). As this low-level/baseline expression of DCX protein is not detectable using immunohistochemistry (Kremer *et al.*, 2013; Gualtieri *et al.*, 2017), its presence in mature neurons within neurogenic areas of the adult avian brain may obscure a relationship between hippocampal *DCX* mRNA levels and numbers of differentiating immature neurons. The insensitivity of baseline expression to experiential factors which modulate AHN, such as exercise (Kremer *et al.*, 2013), may explain why total *DCX* mRNA levels do not reflect the chronic stress arising from severe KBFs. Certain technical control measures may be undertaken to account for these issues in rats and mice, but are not applicable to the avian HF. In the case of birds, AHN is not limited to a discrete hippocampal substructure, equivalent to the DG, or to a single cell population, as with granule cells. It is therefore not possible to perform a micro-dissection of the neurogenic region, or to employ a cell-type specific housekeeping gene for normalization, such as Prox-1 (as in Gualtieri *et al*., 2017). These technical considerations may limit the use of *DCX* mRNA as a proxy for AHN in the chicken brain, and are discussed in more detail in section 6.2.

4.5.5 Conclusions

In commercial laying hens, severe KBFs present 3-4 weeks before sampling are associated with a reduced density of new-born neurons across the HF when compared to flock-mates with minimal KBFs. Additionally, KBF severity scores at this time negatively predict DCX^+ cell counts across individual hens, while in the caudal HF, hens that developed KBFs earlier tend to have fewer DCX^+ multipolar neurons. Further information regarding the duration that KBFs continue to be a source of pain or stress will be necessary in order to determine the time scale in which AHN over the whole HF integrates this painful experience. On the other hand, vertical movement within the aviary 3-4 weeks prior to sampling is not associated with AHN levels. Results suggest that, like the rodent hippocampus, the avian HF is sensitive to the experience of pain as a result of KBFs, on a time scale of at least 3-4 weeks. Downregulated AHN lends support to the notion that KBFs present a source of chronic stress, which is associated with induction of a depression-like state in mammals and thus likely detrimental to the affective experience of commercial laying hens. Management steps to reduce or delay acquisition of KBFs in commercial laying hen systems are thus likely to have a notable impact on animal welfare.

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Chapter 5. Poor Physical Condition is Associated with Lower Hippocampal Neurogenesis in Adult Hens from Enriched Cage and Aviary Commercial Housing

5.1 Abstract

Commercial laying hens are commonly housed in enriched cages, or in cage-free aviaries or barns, which sometimes provide access to outdoor areas. While there are multiple differences between these environments, commonly employed measures of stress, such as levels of corticosterone, have not provided a consistent picture of which alternative is associated with better welfare. Variation between individuals within any system may also be substantial. AHN may present a novel biomarker capable of integrating experiences with positive and negative valence to reflect the sum of stressors associated with particular conditions. Adult hens approaching the end of lay were therefore sampled from a multi-tier aviary with an outdoor range, and an enriched colony cage system, on adjacent sites. Body size, feather cover and redness of the comb were used to select 12 birds in good physical condition and 12 birds in poor condition from each system. Using immunohistochemistry, immature neurons expressing doublecortin (DCX) were quantified in serial hippocampal sections. Additionally, expression of mRNA for inflammatory cytokines was quantified in the spleen and contents of the caecal microbiome were sequenced. DCX^+ cell densities did not differ with housing system, but were lower in hens of poor physical condition than in their good condition counterparts. Splenic levels of interleukin-6 (*IL-6*) mRNA were also higher in good condition hens, while the relative abundance of methanogenic archea in the caecum was higher in poor condition hens from both housing systems. The findings suggest that a relatively greater degree of cumulative chronic stress is experienced by those individual hens in the poorest condition, while a trade-off between positive and negative factors associated with cage-free systems may result in little overall difference from enriched-cage alternatives. However, systems producing the highest proportion of birds in a poor condition may lead to the lowest overall net welfare.

The dataset is stored in a publicly accessible repository, at: https://doi.org/10.25405/data.ncl.14135153.

5.2 Introduction

Subsequent to legislation prohibiting the use of barren battery cages in the European Union (EU Council Directive 1999/74/EC), commercial laying hens are generally housed in enriched (or furnished) cages, or in non-cage aviaries or floor-housing systems (Rodenburg *et al.*, 2008). These housing systems differ in multiple environmental parameters, particularly where cage-free systems also provide access to an outdoor range. Identifying which of these housing alternatives is associated with the highest welfare and lowest cumulative stress has important implications for the egg production industry, due to consumer concern for welfare (Alonso *et al.*, 2020) and negative consequences of stress for production (e.g. Hemsworth and Barnett, 1989; Shini *et al.*, 2009; Lara and Rostagno, 2013). Marked influences of certain discrete aspects of the housing environment for the stress-related behaviour and physiology of laying hens have been demonstrated. For example, providing hens with foraging material in the form of long-cut straw led to shorter durations of tonic immobility (TI), lower heterophil:lymphocyte (H:L) white blood cell ratios, higher egg production and less feather pecking compared to hens housed identically, but without straw (El-Lethey *et al.*, 2000). A multitude of differences that exist between multi-tier aviary systems (especially with outdoor range access) and enriched cage systems might similarly influence overall stressful experience for hens. Notably, the housing systems differ in terms of average group sizes, environmental complexity, exposure to parasites and disease, challenges to bone health/integrity, and opportunities for both a naturalistic behavioural repertoire (e.g. foraging, comfort behaviour & behaviour in the vertical plane) and for deleterious behaviour (e.g. cannibalism, piling and smothering) (Lay Jr. *et al.*, 2011). In terms of consumer perceptions, barn or aviary systems are rated more favourably than caged systems, and free-range systems are valued most highly (Norwood and Lusk, 2011). However, large scale studies have found average on-farm mortality during the laying period to be significantly lower in cages compared to barn, free-range, or organic flocks (Fossum *et al.*, 2009; Weeks *et al.*, 2012). Existing measures of stress have not clearly determined whether or not these alternative housing systems are associated with differences in overall cumulative experience.

Measurements of corticosteroids (CORT) are the most commonly employed physiological parameter for assessing stress in laying hens (Nicol *et al.*, 2009). However, large scale comparisons of alternative housing systems, such as the LayWel project, have produced highly inconsistent results (Shields and Duncan, 2009). Studies comparing conventional (or battery) to enriched (or furnished) cages have variously reported relatively higher levels of CORT in conventional cages (Buil *et al.*, 2006; Pohle and Cheng, 2009), higher CORT in furnished cages

(Guémené *et al.*, 2006), no difference (Guesdon *et al.*, 2004), or a relationship that reversed over the laying period (Buil *et al.*, 2006; experiment 2). Comparisons between caged and non-cage (pen or aviary) housing have yielded similarly mixed results. Basal plasma CORT has been found to be higher in caged than in aviary-housed hens (Colson *et al.*, 2005), higher in floor pens (but not those with range access) than cages (Koelkebeck and Cain, 1984; Koelkebeck *et al.*, 1986; Koelkebeck *et al.*, 1987), and higher in both floor pens and high-density cages than in low- and moderate-density cages (Craig et al., 1986). Mench (1986) found no difference in levels of CORT, or in H:L ratios and antibody titres, once the use of trap-nesting to capture pen birds for sampling was discontinued. Given that CORT levels are raised by acute stress, it is possible that differential stress associated with methods or ease of capture may contribute to these inconsistent findings. It is also notable that plasma CORT levels fail to reflect the negative welfare consequences of decreasing space allowance in cages, which are indicated by reduced productivity and greater mortality (Rushen, 1991; Shields and Duncan, 2009).

It has been argued that other physiological measures, such as the H:L ratio and fluctuating asymmetry, may better reflect long-term exposure to stress (Gross and Siegel, 1983; Parsons, 1990). H:L ratios were found to be higher for hens in battery cages (4 birds per cage) when compared to both modified/enriched cages (10 birds per cage) and an intensive free range system (2200 birds) (Shini, 2003). These measures also suggest that access to an outdoor range attenuates the experience of stress, leading to lower H:L ratios and lower relative asymmetry (of leg length, wattle length, and combined) than for birds also housed in deep litter but without outdoor access (Campo *et al.*, 2008). Comparable TI durations suggested that levels of fear did not differ between the groups. However, another study found no difference in H:L ratios between hens kept in conventional cages (4 birds per cage), modified (enriched) cages (8 hens per cage) and a free-range system (Salamano *et al.*, 2010). Results associated with existing measures considered to be more reliable are therefore also inconsistent.

Housing conditions have also been associated with differences in the behavioural expression of fear. Hens housed in battery cages exhibit longer durations of TI than birds housed in larger floor pens (Jones and Faure, 1981), three different types of aviary (Hansen *et al.*, 1993) or a free range system (Scott *et al.*, 1998). Another study found prolonged durations of TI for hens kept in 17-bird cages compared to hens kept either in floor pens, single bird cages, or 5-bird cages (Kujiyat *et al.*, 1983). Compared to hens housed in aviaries or pens, hens kept in conventional cages react more fearfully to a novel object (Hughes and Black, 1974), explore this less and are less active (Colson *et al.*, 2005). Similarly, when tested in an unfamiliar

environment, a higher proportion of hens from both furnished cages of 60 birds and conventional cages of 3-6 birds remained immobile and oriented towards an approaching human than did their non-cage counterparts (Graml *et al.*, 2008). A further study found longer durations of TI in hens from furnished cages compared to hens from non-cage systems over multiple flocks, with no effect of strain (Rodenburg *et al.*, 2008). These behavioural findings suggest that alternative commercial housing environments are indeed associated with varying degrees of collective stress for hens. A previous study compared cell morphology and innervation patterns in the HF and nidopallium caudolaterale of hens kept in different housing systems (battery cage, small littered ground pen & free range), and found that hens from the free range system developed larger cells in the dorsomedial hippocampus than hens from the battery housing (Patzke *et al.*, 2009). While these findings suggest that adult housing conditions lead to alterations in hippocampal plasticity, consequences for adult neurogenesis have yet to be quantified.

In addition to variation between housing systems, the level of variation in experience between individuals within a system has important implications for welfare and management. Variation between individual laying hens exists in multiple domains, including health (Fossum *et al.*, 2009), susceptibility to disease (Humphrey, 2006) and physical condition (Webster and Hurnik, 1991), responsiveness to stress (Littin and Cockrem, 2001), social status (Rushen, 1982), preferences (Nicol *et al.*, 2009), personality and cognition (e.g. de Haas *et al.*, 2017). These individual differences are likely to equate to variation in chronic stressful experience within any type of commercial housing system. The stress response is activated by threats to homeostasis or well-being (Herman *et al.*, 2016), and prolonged stress has phenotypic consequences, such as suppressions in weight gain (Gross and Siegel, 1981). The physical condition of a bird may therefore reflect the degree of stress it has experienced, with poor body condition potentially comprising both a cause and a consequence of chronic exposure to stress. Several externally observable characteristics may reflect the physical condition of an individual bird. Firstly, body weight in chickens is reduced by the administration of CORT (El-lethey *et al.*, 2001; Eid *et al.*, 2010) or adrenocorticotropic hormone (Thaxton and Puvadolpirod, 2000), by immune challenge (Yang *et al.*, 2011) and by stressful experiences, such as transport (Karaman, 2009). Hens of lower body weight may therefore have experienced relatively more stress.

Secondly, damage to the plumage is associated with a reduced thermoregulatory capacity (Herremans and Decuypere, 1986) and has been linked to indicators of stress and fear. For example, poor plumage condition is associated with relatively higher fluctuating asymmetry

(Campo and Prieto, 2009) and H:L ratios (Campo *et al.*, 2001). Hens with poorer feathering are more nervous and evasive in response to an observer in front of the cage (Ouart and Adams, 1982), and a negative correlation between nervousness and feather scores exists for individual hens (Na-Lampang and Craig, 1990). One study recorded longer durations of TI with poorer plumage condition in 30-week-old aviary-housed hens (Hansen *et al.*, 1993), though another found very poorly feathered hens (from pens of 90 birds) to exhibit shorter TI than hens with perfect plumage (Campo *et al.*, 2001). Braastad and Katle (2007) selectively bred hens for high or low efficiency of food consumption, and found that low-efficiency hens had poorer plumage and engaged in more escape and aggressive behaviour, while the poorer the plumage the more behavioural agitation an individual hen exhibited. This suggests that plumage condition may be an indicator of metabolic and behavioural characteristics. Poor feather cover and lower body weight are both significant risk factors for mortality during transport to the slaughter plant at the end of lay (Weeks *et al.*, 2012), and may thus reflect generally poor welfare.

Social status may be a source of stress for subordinate hens, as these individuals are often threatened and attacked by more dominant birds (Choudary and Craig, 1972; Gibson *et al.*, 1988) and may be restricted in their access to resources (Hughes, 1983; Shimmura *et al.*, 2008). Aggressive pecks are used by hens to maintain the dominance hierarchy (Rushen, 1982), and hens receiving greater numbers of aggressive pecks had both increased feather damage and decreased body weight at 27 and 32 weeks of age (Bilcik and Keeling, 1999), while the number of severe feather pecks received predicted feather damage at all ages. Stress also triggers alterations in blood supply, which influence comb colour in hens (O'Connor *et al.*, 2011). Comb colour (scored on a scale of 1 = dark red to 6 = light pink) is positively correlated with dominance rank (Bradshaw, 1992b), and individuals with darker combs tend to defeat opponents with paler combs (Cloutier *et al.*, 1996). Flocks of hens that displayed higher levels of fear were also found to have lighter combs than less fearful flocks (Bestman and Wagenaar, 2014). Sampling hens with extremes of feather cover, body weight and comb colour might therefore comprise an approximate method of selecting individuals from each end of the "pecking order", without undertaking time-consuming behavioural observations.

Finally, in cage-free systems that provide access to an outdoor area, frequency of range use varies greatly between individual birds (e.g. Richards *et al.*, 2011; Gebhardt-Henrich *et al.*, 2014). Indoor-preferring birds may be more fearful, as they exhibit longer durations of TI (Grigor *et al.*, 1995; Hartcher *et al.*, 2016), are more vigilant and slower to feed following playback of an alarm call (Campbell *et al.*, 2019), produce fewer vocalisations during manual restraint, and move less in open field tests (Campbell et al., 2016). Comb colour has also been

associated with ranging behaviour, as hens with darker combs and better condition beaks roam further from the shed (Larsen *et al.*, 2018). This research suggests that less frequent use of the range may be associated with greater stress for individual hens.

In rats and mice, rates of AHN reflect long-term experiences in a quantitative manner. Combined stressors, such as pain and stress, have an additive effect in suppressing AHN levels (Romero-Grimaldi *et al.*, 2015), while concurrent positive experiences ameliorate declines in numbers of new neurons (e.g. pain and exercise/enrichment (Zheng *et al.*, 2017), exercise and stress (Nakajima *et al.*, 2010; Kiuchi *et al.*, 2012), stress and sexual activity (Kim *et al.*, 2013)). Sequential experiences of shared valence also have additive effects on AHN (Fabel *et al.*, 2009), while effects of stress are offset when followed by enrichment (Veena *et al.*, 2008; Veena *et al.*, 2009). In laying hens, the suppression of AHN by long-term negative experience associated with unpredictable chronic mild stress (Gualtieri *et al.*, 2019) and severe keel bone fractures (Armstrong *et al.*, 2020) has also been demonstrated. As such, relative levels of differentiating immature neurons in the HF of hens at the end of lay may reflect the net sum of relatively long-term stressors and positive affective experiences in different commercial housing systems.

Given that alternative commercial housing systems for adult laying hens differ in numerous ways, it was hypothesised that these living conditions are associated with differing levels of cumulative stress and positive experiences. Due to the greater environmental complexity and behavioural freedom afforded, it was predicted that levels of AHN at the end of lay would be higher in hens from a multi-tier aviary with an outdoor range than in hens from an enriched cage system. Additionally, it was hypothesised that individual hens with poorer body conditions experience relatively more stress during their time in the adult housing than flock-mates in better condition. It was predicted that hens with low body mass, poor feather cover and pale combs from both housing systems would have relatively lower AHN when compared to hens displaying the opposite phenotypic characteristics. To determine the contribution of this inter- and intra- system variation to the net valence of long-term experience, densities of immature neurons expressing doublecortin (DCX) in serial hippocampal sections were compared between these groups of commercial hens. As an attempt to identify factors that contribute to, or mediate, differential stressful experience, compositions of caecal microbiota and inflammatory gene expression in the spleen were also measured.

5.3 Methods

5.3.1 Ethical statement

This study was approved by the Animal Welfare and Ethical Review Body at Newcastle University (Project ID #702). Feed and water were provided *ad libitum* in holding pens during the short time that hens were kept at the university.

5.3.2 Adult hens

From one day old, H&N and Hy-Line Brown pullets were reared in two floor-based systems with litter at farms in Shropshire, UK. Both sites were operated by the same pullet rearing company, according to RSPCA-Assured standards, and arrived at an egg production farm in the north-east of England in October 2017. The H&N pullets were 16 weeks old when introduced to the multi-tier free range adult housing system, whilst the Hy-Line birds were 17 weeks old upon introduction to the enriched colony cage system on the adjacent site. By the time of sampling in October 2018, both groups had spent almost a year living in their respective systems, and hens were 65 weeks old in the multi-tier aviary system and 66 weeks old in the enriched-cage system.

The multi-tier aviary housing unit consisted of 16,000 birds, divided into four internal flocks of 4,000 hens. However, as these birds all shared a range, individuals could move between the flocks during the day by accessing different popholes. Within the shed, the floor was covered with litter in the form of wood shavings and there were three additional tiers, the top of which was located 2.4 m above the floor. The system provided round metal perches and nest boxes shaded by orange plastic dividers. Internal stocking density was 13.3 birds/m^2. Water was provided through nipple-drinkers, with one nipple for every 10 birds. Layer feed was circulated through a conveyor belt system, with a frequency of eight times per day. The average temperature inside the shed was 19 – 22ºC. Birds received 15.75 hours of daylight per day, from 06:45 to 22:30. Popholes opened at 09:00 each day and were closed 30 minutes after twilight ended. The grassy range had a dimension of 20 acres and contained several two-tiered, covered wooden shelters, with ramps to the upper tier. No cover was provided by vegetation.

The enriched colony cage housing unit contained 33,120 birds, with a stocking density of 15 birds/m^2. Each cage was 300 (l) x 150 (w) x 55 (h) cm in dimension and held 50 birds, providing 750 cm^2 space per bird. Cages were arranged into four columns of three vertical tiers with 22 cages per row, repeated over two floors. Enrichment provided in each cage consisted of perches, a nest box, a scratch mat and a grit auger to drop feed onto the scratch mat. The average

temperature in the unit was 19 – 23ºC. Birds received 15.5 hours of daylight per day, from 01:45 to 17:15. Water was provided through nipple-drinkers, with one nipple for every 10 birds. Layer feed was circulated through a conveyor belt system, with a frequency of six times per day.

Birds in both systems experienced an identical programme of vaccinations. Hens in the multi-tier system were given a wormer at four intervals during lay. Due to poor laying performance, colony birds alone were given a course of antibiotics at 39 weeks of age (Denegard; for the treatment and prevention of chronic respiratory disease and air sacculitis caused by *Mycoplasma gallisepticum* and *Mycoplasma synoviae*). Feed intake was typically 20 g per bird higher per day in multi-tier system than in the enriched cages. At 65 weeks of age (for comparability), average bird weight was 1544g in the multi-tier system compared to 1967g in the enriched-cage system. Production rates were 90.3% in the multi-tier system and 81.6% in the enriched cages, and cumulative mortality had reached 2.50 and 2.74% respectively.

5.3.3 Sampling

In order to compare high and low welfare individuals within each system, birds with good and poor external indicators of physical condition were selected. Hens were chosen by the farm's production manager, who was familiar with the conditions of the birds. The criteria employed were: *i*) feather coverage, *ii*) bodyweight, and *iii*) redness of the comb and face, wherein a high level of each factor represented good physical condition. Because keel bone damage was previously found to be associated with reduced AHN (Chapter 4), initially selected hens were palpated, and those displaying signs of damage were rejected.

A total of 12 hens in good physical condition and 12 hens in poor physical condition were sampled from both the multi-tier aviary and enriched-cage system, equating to a total sample size of 48 birds. Sampling occurred over four successive days, upon each of which 12 birds (3x multi-tier/good condition, 3x multi-tier/poor condition, 3x enriched-cage/good condition and 3x enriched-cage/poor condition) were selected from the farm and transported in carry boxes to Newcastle University for processing on the same day. In an attempt to capture extremes of experience in the multi-tier system, good condition birds were selected directly from the range, while poor condition birds were selected from the top inside tier. This meant that the sampled good condition birds ventured outside at least some of the time, whilst the sampled poor condition birds may or may not choose to range. To allow birds time to go outside, there was a 30-minute delay between the opening of pop-holes in the morning and sampling of birds from the range. Hens were sampled from a different internal sub-flock of the shed on each of the four

days, along with the proximal area of the range (Figure 5.1a). To ensure independence in the colony system, no two birds were sampled from the same cage, but a good and poor condition bird were always selected from different cages on the same row. In order to collect a sample representative of the whole housing system, birds were sampled from each vertical tier (top, middle and bottom) in both an inside and outside column on each of the two floors (Figure 5.1b). Representative images of good and poor physical condition hens sampled from each housing system are displayed in Figure 5.2.

a)

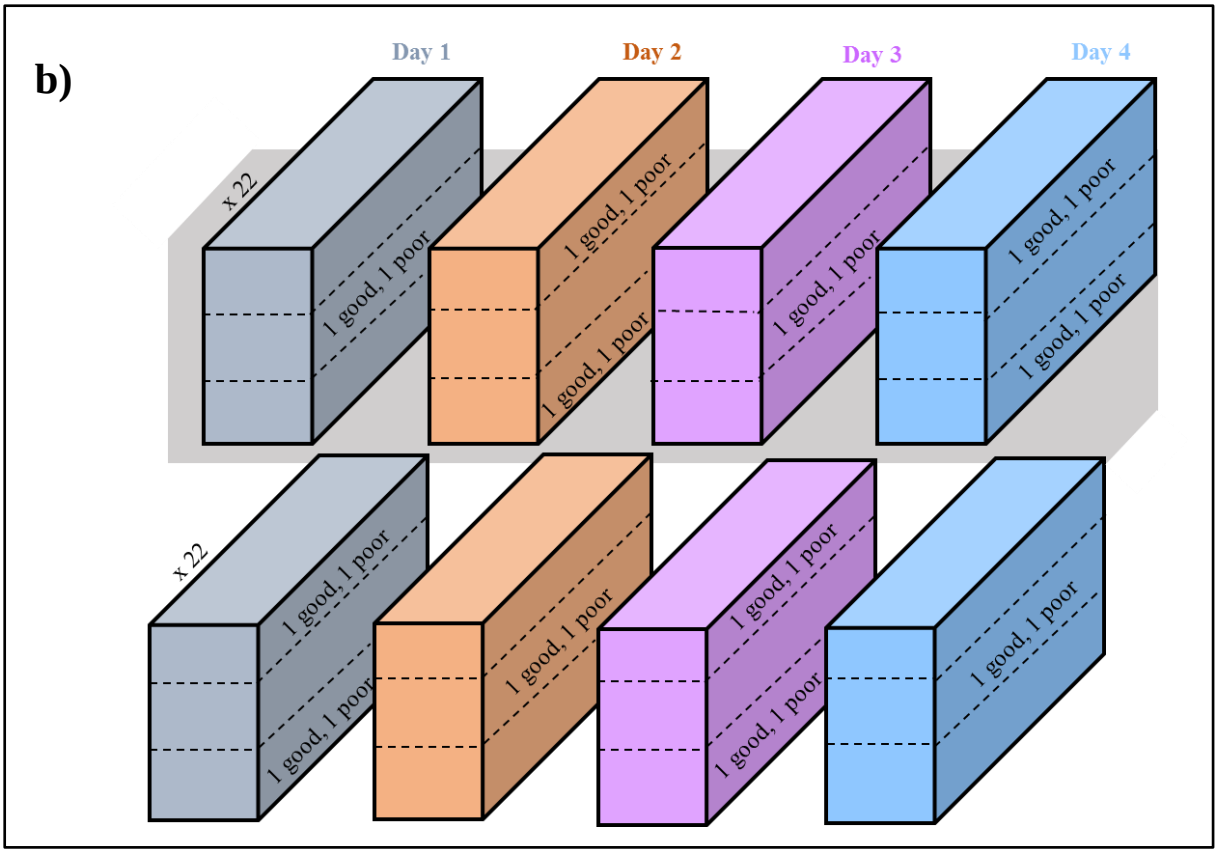

Figure 5.1. Schedule for representative sampling of hens of good and poor physical condition from a) the multi-tier aviary with an outdoor range and b) the enriched colony cage system, over four days on which tissue was collected.

Figure 5.2. Representative images of good and poor physical condition hens sampled from each housing system. Colony refers to the enriched colony cage system, while the multi-tier aviary provided access to an outdoor range.

5.3.4 Sampling of pullets from the rearing farm

As different strains of brown hen were sampled from the two adult housing systems, pullets of the same genotypes were also sampled directly from the rearing farm, in order to provide a baseline for other measures taken. H&N and Hy-Line pullets were housed from one day-old in adjacent barns of a rearing farm in Shropshire (Country Fresh Pullets, UK), according to RSPCA Assured standards. The chicks originated from separate (hybrid-specific) hatcheries, each in the west of England. Both housing sheds contained litter in the form of woodshavings (Easichik, UK), which were bedded to a depth of 3" at the point chicks were introduced. Raised slatted areas were provided for perching, with access assisted by ramps placed every 20 to 30 feet along their length. The H&N shed had a total area of 1,187.92 m^2 (including the floor and raised slatted areas) and contained 17,085 pullets, which equated to a stocking density of 14.4 birds/m^2. Though similar in design, the Hy-Line shed had a larger total area of 1,722.12 m^2 and housed 25,236 pullets, with a stocking density of 14.7 birds/m^2. Water was provided via nipple drinkers, with 12.2 and 12.4 birds per drinker in the H&N and Hy-Line sheds respectively. Both sets of birds had access to feed at all times via a chain feeder, but the quantity of feed in the feeder and the number of times it was topped up per day was adjusted throughout rearing, in order to maintain target bodyweight for each flock. The H&N shed housed 39 birds per metre of chain feeder, compared to 37 birds in the Hy-Line shed. Both strains received 10 hours of light per day from six weeks of age onwards, with a brightness of 10 lux at bird height. Ambient temperatures were 32-33ºC for at the introduction of day-old chicks and were gradually reduced by 0.5ºC per day, before being maintained at 20ºC. Both strains experienced an identical program of vaccinations, administered from day old to 13 weeks. At the time of sampling, average bird weight was 1203g for the H&N flock and 1239g for the Hy-Line flock. Both flocks had a cumulative mortality of 1.9%.

Sampling of pullets occurred during a single day, when both strains of bird were 14 weeks and 3 days old. Twelve birds of average size and physical condition were selected from each rearing barn (total n=24) and manually palpated to determine if keel bone fractures were present. Individuals exhibiting damage were avoided. Animals were placed in carry boxes and transported to Newcastle University, where they were housed in two pens overnight (one Hy-Line, one H&N) prior to tissue collection the following day.

5.3.5 Tissue collection & processing

Collection of tissue from the adult hens and pullets occurred in two separate phases (in October 2018 and January 2019, respectively). Animals were weighed before being killed with an

intravenous injection of pentobarbital (Euthatal), according to a schedule that alternated between housing systems and physical conditions for the adult hens (n=48) and genotypes for the pullets (n=24). Immediately thereafter, the head was removed and blood samples were immediately collected from the vessels of the neck using heparinised capillary tubes. Blood was centrifuged in 1.5 ml Eppendorf tubes at 5,000 rpm for ten minutes, before the plasma layer was separated from red blood cells with a pipette and both samples were frozen on dry ice. Samples were later shipped on dry ice for epigenetic analysis by collaborators in Per Jensen's research group (results to be reported elsewhere). The spleen was removed and weighed before a sample was placed on ice in a tube containing 1 ml RNAlater® Stabilization Solution (Thermo Fisher Scientific, Loughborough, UK). The caecum was dissected from the gut and placed into a 15ml Falcon tube before freezing on dry ice.

Simultaneously, brains were removed from the skull, placed into 0.1 M PBS in a Petri dish and divided along the longitudinal fissure with a scalpel. The forebrain hemisphere collected for immunohistochemical analysis alternated between left and right, in a manner that was balanced within groups of hens of each physical condition and from each housing system. This tissue was immersion fixed for 44-48 h in 4% paraformaldehyde in 0.5 M PBS at 4°C. Samples were then cryoprotected in a solution of 30% sucrose in 0.5 M PBS, before being embedded in OCT (4583, Electron Microscopy Sciences - USA). Coronal sections (50 μm) were cut on a cryostat (HM 550, Microm – Germany) and stored in cryoprotectant solution (30% glycerol, 30% ethylene glycol, 0.1M PBS) at -80ºC. Serial sections taken at 400μm intervals were processed for immunohistochemistry.

5.3.6 Immunohistochemistry & quantification of AHN

As previously, sections were stained using an antibody for doublecortin (DCX) to allow quantification of differentiating immature neurons generated through AHN. Free-floating sections from the adult hens were stained over six batches, each of which contained tissue from eight birds (two x multi-tier/good condition, two x multi-tier/poor condition, two x enriched-cage/good condition and two x enriched cage/poor condition). Sections from the pullets were stained over three batches, each of which contained tissue from eight birds, wherein four were of the H&N strain and four were Hy-Line. Staining was conducted according to the protocol detailed in section 4.3.7. This again utilised rabbit polyclonal to doublecortin primary antibody (Abcam Cat# ab18723, RRID:AB_732011) at a concentration of 1:1000, and biotinylated anti-rabbit IgG secondary antibody, made in goat (Vector Labs, BA-1000), at a concentration of 1:500.

Stained DCX^+ multipolar and bipolar cells were quantified in the rostral (interaural 5.68/0.50) and caudal (interaural 0.50/-0.50) HF, using the equipment and parameters detailed in section 4.3.8. For each animal, 4 to 6 hippocampal sections 800 μm apart were again systematically analysed, starting with the rostral-most section bearing hippocampal tissue. Image analysis was performed with Stereo Investigator software (version 2018.1.1, MBF Bioscience, USA), with HF borders outlined at 2.5X magnification (0.07 numerical aperture) and cell counting performed at 100X magnification (0.65 numerical aperture) according to the Optical Fractionator method. Densities of DCX^+ cells per cubic millimetre of sampled tissue were calculated by dividing the number of counted cells of each type by the area of the counting frame (2500μm), multiplying by both the number of counting sites sampled in that brain and the section thickness (50μm), and multiplying by 10^9. The rostral and caudal hippocampus were treated separately.

5.3.7 Inflammatory gene expression

RNA was extracted from the sampled spleen tissue and reverse transcribed into cDNA according to the protocol described in section 2.3.6. Gene-specific templates for standard curves were produced according to the method described in section 2.3.7, with primer sequences for target genes involved in the inflammatory response (*IL1β*, *IL6*, *IL8*, *IL10* & *TGFβ*) displayed in Table 5.1. Quantitative PCR (qPCR) assays were conducted as in section 2.3.8. As before, *LBR* was used as a control gene for normalisation. The 48 samples from the adult hens were processed in a single batch. Template production and qPCR assays were conducted by student Lucy Addison as part of her BIO3199 research project. Gene expression values were log(10)-transformed for statistical analysis.

Table 5.1. Primer sequences employed for quantification of inflammatory cytokine expression in splenic tissue.

Gene	Accession	Orientation	Primer Sequence (5'-3')	Product Length (base pairs)
LBR	NM_205342	Forward Reverse	GGTGTGGGTTCCATTTGTCTACA CTGCAACCGGCCAAGAAA	80
IL1β	NM_204524.1	Forward Reverse	TGCCTGCAGAAGAAGCCTCG CTCCGCAGCAGTTTGGTCAT	137
IL6	NM_204628 .1	Forward Reverse	TCGCCTTTCAGACCTACCTG CAGATTGGCGAGGAGGGATT	179
IL8	NM_204608 .1	Forward Reverse	TGTGAAGAGATCGCTGTGTG AGGCATCGCATTCCAGC	85
IL10	NM_001004414.2	Forward Reverse	GGGAGCTGAGGGTGAAGTTT TCTGTGTAGAAGCGCAGCAT	154
TGFβ	NM_001318 456.1	Forward Reverse	TTACTACGTGGGCCGGAATG CCCCCAAAAAGGGAACCATCT	193

5.3.8 Composition of the caecal microbiome

Analysis of the caecal microbiome was conducted externally, by collaborators Peter Richards and Paul Wigley at the University of Liverpool. The caeca are frequently utilised as a site for the investigation of microbiota due to their involvement in processes of immune maturation and metabolism (Richards *et al*., 2019). The methods employed for these analyses were recently applied to characterization of development of the caecal microbiome in broiler chickens, and are described in detail in Richards *et al*. (2019). Briefly, a sterile scalpel was used to open the caecum longitudinally and gently scrape off the mucus layer, which was transferred to a sterile container. Mucus samples were weighed, diluted with sterile water and homogenized. DNA was extracted using the Zymobiomics DNA MiniKit (Cambridge Bioscience, UK), according to the manufacturer's instructions. Extracted DNA was quantified using a Qubit dsDNA HS fluorometric kit (Invitrogen). Paired-end sequencing of the 16S rRNA gene was conducted at the Centre for Genomic Research (University of Liverpool) through an Illumina MiSeq run. The V4 hypervariable region (515F/R806) was amplified to yield an amplicon of 254 base pairs (Caporaso *et al*., 2011), and subsequent amplification was performed using a two-step PCR with a HiFi Hot Start polymerase (Kapa) (D'Amore *et al*., 2016). Primer sequences are reported in Richards *et al*. (2019). QIIME2 version 2018.4.0 was used for analysis of the Illumina data (Bolyen *et al*., 2018), and amplicon sequence variant (ASV) assignment was performed with the dada2 plugin (Callahan *et al*., 2017). Taxonomy was assigned using the q2-feature-classifier plugin, with a pretrained NaiveBayes classifier based on the SILVA 132 database of the 515F/R806 region of the 16S rRNA gene (Yilmaz *et al*., 2014). Gneiss analysis was used to compare the differential relative abundance of groups of microbiota ASVs at the level of order, family, genus and species.

5.3.9 Data analysis

In the sample of pullets, body masses of the two strains were compared through an independent samples t-test. For spleen mass, univariate ANOVAs were conducted with body mass as a covariate and strain as a between-subject fixed factor. In the adult hens, a univariate ANOVA was used to compare body masses, with housing system and physical condition as fixed factors and together in an interaction term. Spleen mass was explored in a similar model, but which also included body mass as a covariate. To explore AHN in the laying hen pullets, separate linear mixed models (LMMs) with unstructured covariance were conducted for raw DCX^+ multipolar and bipolar cell densities. These included staining batch as a random factor, HF subregion (rostral/caudal) as a repeated fixed factor, and strain (H&N/Hy-Line) as between-subject fixed factor. The interaction between subregion and strain was also included. To analyse

cell counts for the adult hens, separate LMMs were again conducted with DCX^+ multipolar and bipolar cell densities as the dependent variables. Models included staining batch as a random factor, HF subregion as a repeated fixed factor and housing system (multi-tier free range/enriched cage) and physical condition (good/poor) as between-subject fixed factors. Factorial interactions between HF subregion, housing system and condition were explored. Quantified expression of mRNA for inflammatory markers in the spleen was analysed in a series of univariate ANOVAs, with target transcript (*IL10*, *IL1B*, *IL6*, *IL8* or *TGFB*) as the dependent variables. All models included *LBR* mRNA expression as a covariate, alongside housing system and physical condition as between-subject fixed factors and in an interaction term.

For analysis of the caecal microbiome, a chi-square test was used to assess the distribution of taxa across Gneiss analysis groups. Differential abundance in relation to housing system and body condition were explored separately. The proportion of total ASVs classified as being *i*) more abundant in housing system or body condition one, *ii*) more abundant in housing system or body condition two, and *iii*) not differentially abundant, was calculated and applied to the total number of ASVs assigned to each taxa, in order to generate an expected number of each taxa for each group. P values were initially corrected for multiple tests using a false discovery rate (FDR) correction, but as no taxa were significantly overrepresented in any category using the corrected p-values, the uncorrected p-values were used. This analysis was conducted externally, by Peter Richards at the University of Liverpool.

5.4 Results

5.4.1 Body & spleen mass

Body masses of the 24 sampled pullets were consistent with flock averages at the time of sampling. Mean spleen masses are also displayed in Table 5.2.

Table 5.2. Descriptive statistics for body and spleen masses of Hy-Line and H&N pullet strains.

	Hy-Line (n=12)		H&N (n=12)	
	M	SD	M	SD
Body mass (kg)	1.28	0.06	1.23	0.08
Spleen mass (g)	2.74	0.58	2.94	1.18

There was a trend towards the sampled Hy-Line pullets being heavier than their H&N counterparts ($t_{22} = 1.77$, $p = 0.090$). Body mass did not predict spleen mass on an individual basis ($F_{1,21} = 0.681$, $p = 0.419$), and there was no difference in spleen mass between the two

strains ($F_{1,21}$ = 0.624, p = 0.438). One H&N bird had a particularly large spleen, weighing 6.46g. However, after removal of this outlier, neither body mass nor strain came to predict spleen mass (body mass: $F_{1,20}$ = 0.053, p = 0.821; strain: $F_{1,20}$ = 0.338, p = 0.568).

Body and spleen masses of good and poor condition adult hens sampled from the two commercial housing systems are displayed in Figure 5.3.

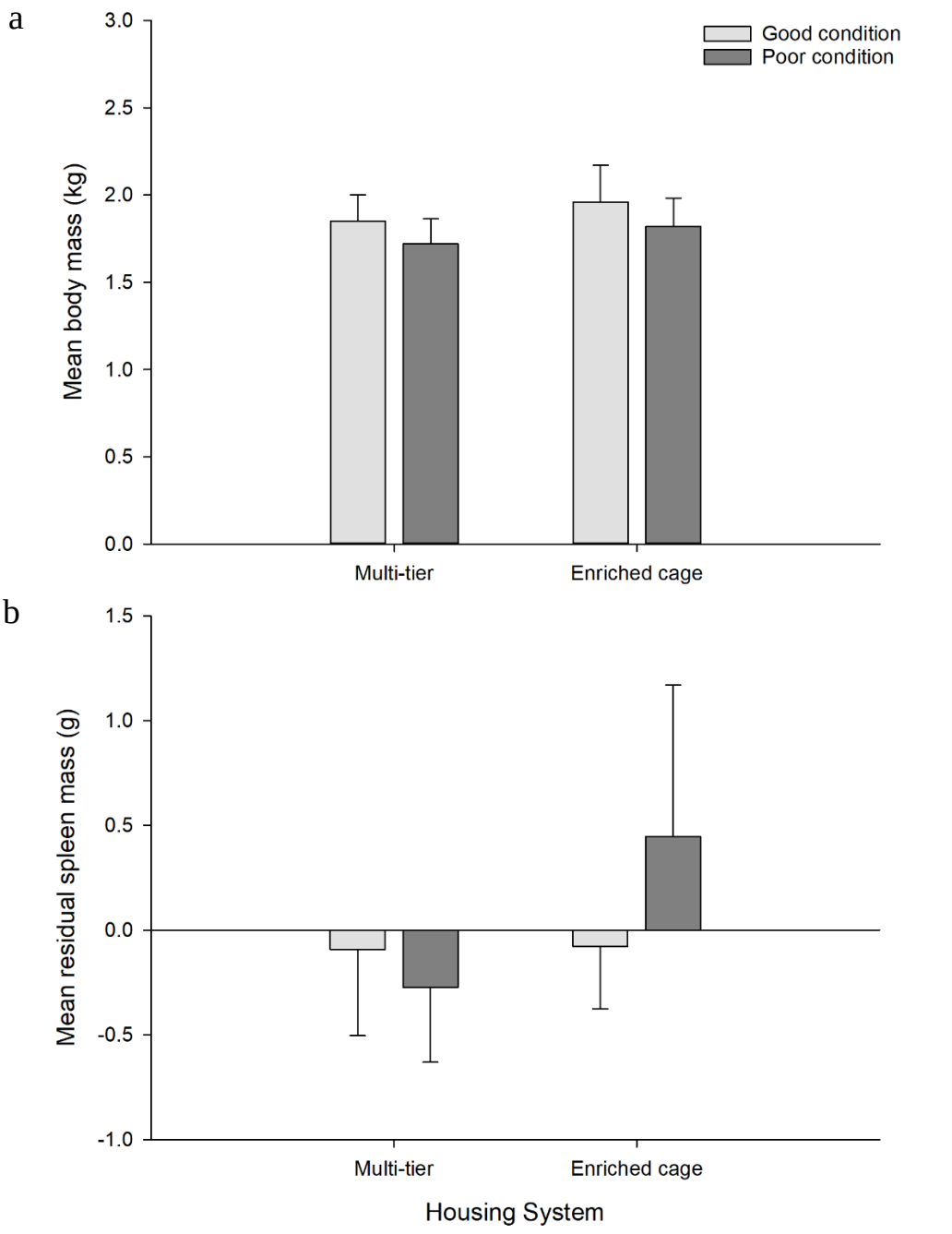

Figure 5.3. Mean a) body and b) spleen masses of adult hens in good and poor physical condition from a multi-tier aviary system with an outdoor range (strain = H&N) and an enriched cage system (strain = Hy-Line). Error bars indicate +1 standard deviation. Spleen masses are residual, after controlling for body mass in a simple linear regression. n = 12 for each sub-group.

As intended, there was a main effect of physical condition on body mass, with good condition hens being heavier than their poor condition counterparts ($F_{1,44}$ = 7.67, p = 0.008). The effect of housing system (or strain) on body mass was also significant, with H&N birds from the multi-tier system having lower mass than Hy-Line birds from the enriched cages ($F_{1,44}$ = 4.50, p = 0.040). There was no interaction between condition and housing system/strain ($F_{1,44}$ = 0.024, p = 0.877).

In the adult hens, body mass significantly predicted spleen mass as a covariate ($F_{1,43}$ = 5.81, p = 0.020). There was no main effect of physical condition on spleen mass ($F_{1,43}$ = 1.07, p = 0.307), but a significant effect of housing system/strain, whereby Hy-Line caged birds had heavier spleens ($F_{1,43}$ = 6.74, p = 0.013). Condition also interacted with housing/strain ($F_{1,43}$ = 6.41, p = 0.015). Specifically, within the enriched-cage system alone, poor condition birds had heavier spleens than good condition birds (p = 0.018), whilst the former group had higher spleen mass than hens of equivalent condition in the multi-tier system (p = 0.001). In contrast, no difference in spleen mass was present between housing systems for birds in good physical condition (p = 0.895).

5.4.2 Adult hippocampal neurogenesis

5.4.2.1 Pullets of two genotypes

For the 14-week-old laying hen pullets, a greater volume of DCX^+ multipolar ($F_{1,22.1}$ = 29.2, p < .001) and bipolar ($F_{1,21.3}$ = 6.21, p = 0.021) cells were quantified in the caudal HF that in the rostral subregion. The H&N and Hy-Line pullet strains did not differ from each other in their densities of DCX^+ multipolar cells over the whole HF ($F_{1,19.4}$ = 0.242, p = 0.628), and there was no interaction between genotype and HF subregion ($F_{1,22.1}$ = 0.541, p = 0.470, Figure 5.4a). Densities of DCX^+ bipolar cells also did not differ between the two strains ($F_{1,21.0}$ = 0.219, p = 0.645), and strain did not interact with HF subregion to predict bipolar densities ($F_{1,21.3}$ = 0.437, p = 0.516, Figure 5.4b).

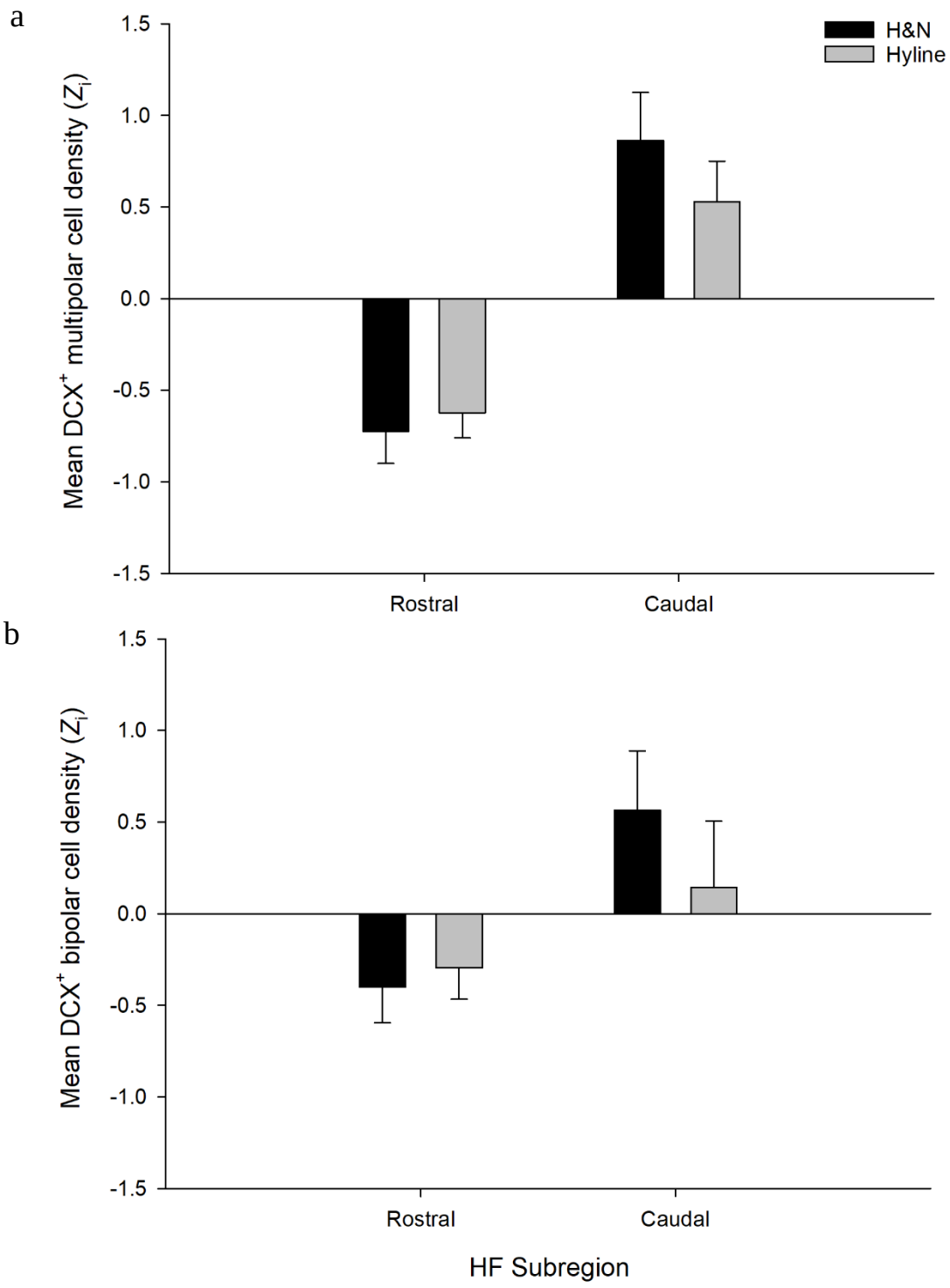

Figure 5.4. Mean densities of a) multipolar and b) bipolar cells expressing doublecortin (DCX) in the rostral and caudal HF subregions of H&N and Hy-Line strain laying hen pullets (14-weeks-old). Error bars display +1 standard error. Cell densities are normalized within staining batches using the standard score (Z_i).

5.4.2.2 Adult hens from two housing systems

For all sampled adult birds, a higher density of DCX^+ multipolar neurons was found in the caudal HF than in the rostral subregion ($F_{1,41.5} = 57.07$, $p < 0.001$). There was no main effect of housing system on multipolar cell density ($F_{1,38.9} = 0.176$, $p = 0.677$), but hens of poor physical condition had lower multipolar cell densities over the whole HF than hens of good physical condition ($F_{1,39.0} = 4.36$, $p = 0.043$, Figure 5.5a). There was no interaction between housing system and physical condition ($F_{1,38.9} = 0.040$, $p = 0.842$), nor did HF subregion interact with housing system ($F_{1,41.6} = 0.684$, $p = 0.413$) or physical condition ($F_{1,41.5} = 1.45$, $p = 0.236$). Finally, there was no three-way interaction between HF subregion, housing system and condition for multipolar cell densities ($F_{1,41.6} = 0.454$, $p = 0.504$).

The density of DCX^+ bipolar cells was also higher at the caudal HF pole than in the rostral region ($F_{1,41.6} = 53.00$, $p < 0.001$). Housing system also had no main effect on bipolar cell densities ($F_{1,38.6} = 0.132$, $p = 0.718$). There was a trend towards hens of poor physical condition having a lower density of DCX^+ bipolar cells than their good condition counterparts ($F_{1,38.7} = 3.32$, $p = 0.076$, Figure 5.5b). There was no interaction between housing system and physical condition ($F_{1,38.6} = 0.338$, $p = 0.564$), and HF subregion did not interact with housing system ($F_{1,41.7} = 0.211$, $p = 0.649$) or physical condition ($F_{1,41.6} = 1.86$, $p = 0.180$). There was also no three-way interaction between HF subregion, housing system and condition on bipolar cell densities ($F_{1,41.7} = 0.730$, $p = 0.398$).

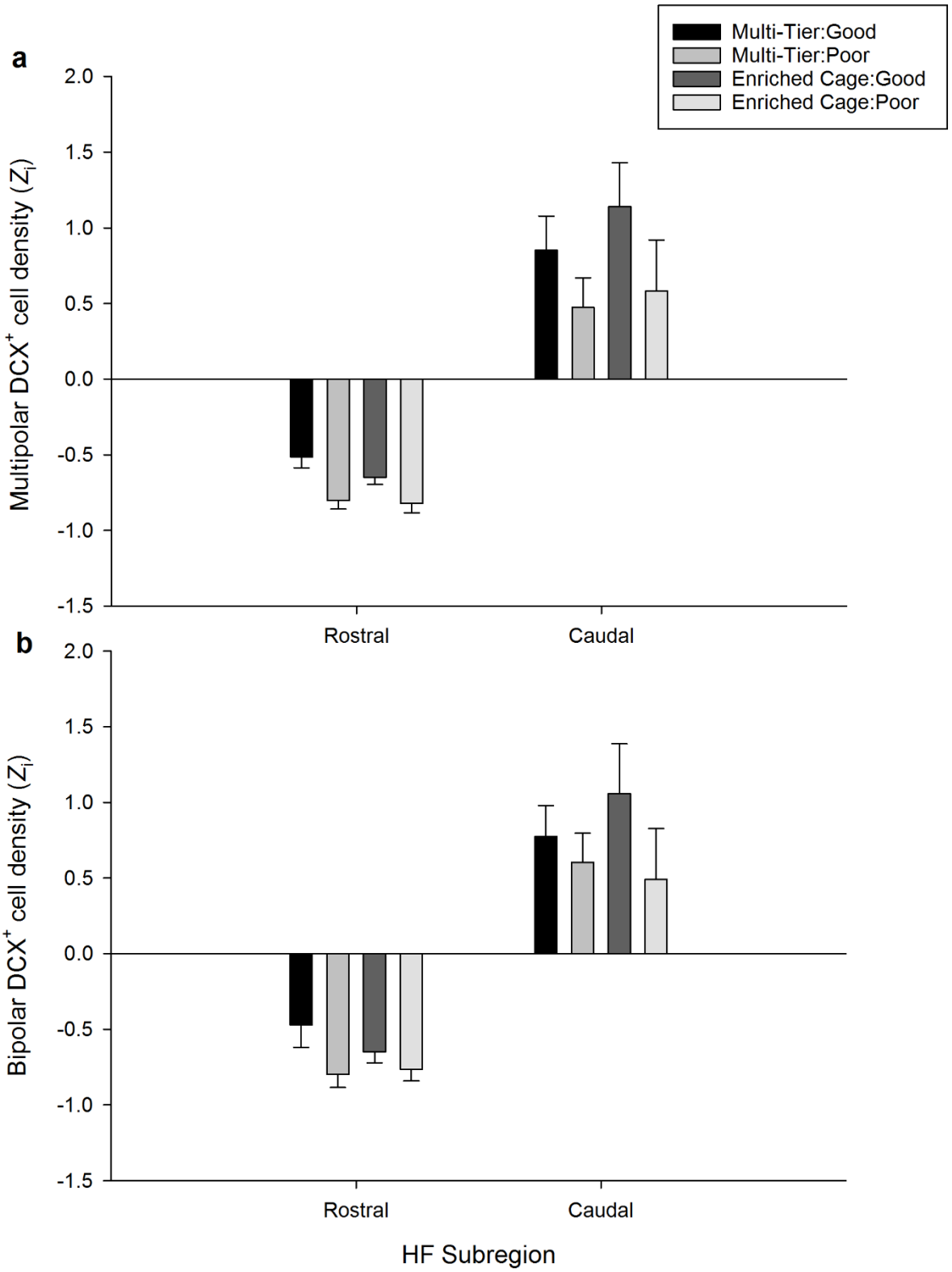

Figure 5.5. Mean densities of cells expressing doublecortin (DCX) with a) multipolar, and b) bipolar (or fusiform) morphology, in the rostral and caudal HF subregions of adult commercial laying hens, grouped according to housing system (multi-tier free range versus enriched cage) and physical body condition. Error bars display +1 standard error. Cell densities are normalized within staining batches using the standard score (Z_i).

5.4.3 Expression of mRNA for inflammatory cytokines in the spleen

Expression of control gene *LBR* in the spleens of the adult hens did not differ with housing system ($F_{1,44}$ = 0.81, p = 0.372) or physical condition ($F_{1,44}$ = 0.006, p = 0.936), nor did these factors interact ($F_{1,44}$ = 0.22, p = 0.641). Inflammatory gene expression also did not differ between the two housing systems for *IL-10* ($F_{1,41}$= 0.13, p = 0.716), *IL-1β* ($F_{1,41}$ = 0.02, p = 0.893), *IL-6* ($F_{1,42}$ = 0.48, p = 0.490), *IL-8* ($F_{1,40}$ = 0.71, p = 0.405), or *TGFβ* ($F_{1,41}$ = 0.52, p = 0.476). There was no effect of physical condition on expression of *IL-10* ($F_{1,41}$= 0.31, p = 0.584), *IL1β* ($F_{1,41}$ < 0.001, p = 0.997), *IL-8* ($F_{1,40}$ = 3.16, p = 0.083), or *TGFβ* ($F_{1,41}$ = 0.02, p = 0.883). However, splenic expression of *IL-6* mRNA was higher in hens in good physical condition (M = -15.77, SEM = 0.066) than in hens in poor physical condition (M = -16.01, SEM = 0.065, $F_{1,42}$ = 6.68, p = 0.013, Figure 5.6). There were no significant interactions between housing and physical condition ($p \geq 0.605$).

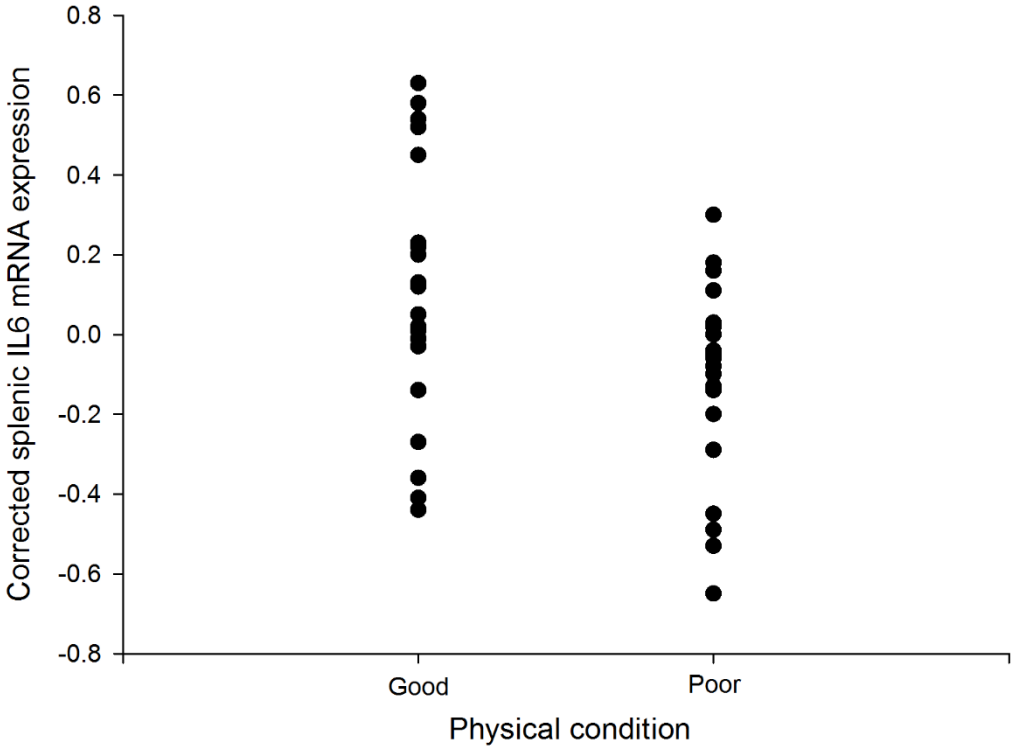

Figure 5.6. Expression of mRNA for interleukin-6 (*IL-6*) in the spleens of adult laying hens in good versus poor physical condition. Values are residual, after correcting for LBR expression and housing system in univariate ANOVAs.

When included as a covariate in the previous model, residual expression of *IL-6* (after controlling for *LBR*) did not correlate with DCX^+ multipolar cell densities in the HF of individual hens ($F_{1,34.4}$ = 0.30 = 0.590). The same was true for bipolar cells ($F_{1,33.9}$ = 1.13 = 0.295).

5.4.4 Composition of the caecal microbiome

Initially, ASVs were filtered to exclude those with a low frequency of less than 57, reducing the number of ASVs included in the analysis from 2,551 to 1,283. Accounting for housing system and physical condition, the overall linear regression model fit of the Gneiss analysis was $R^2 = 0.22$, wherein 17.6% of variance was explained by housing system and 4.1% was explained by physical body condition. At the level of order, *Clostridiales* were significantly underrepresented in samples from multi-tier hens, while *Bacteroidales* and *Spirochaetales* were significantly overrepresented in these samples, when compared to samples from enriched cage hens. However, it should be noted that the caecum is subject to the constant introduction of environmental bacteria by reflux from the urodeum and cloaca, and these are ingested from litter, feed and water (Richards *et al*., 2019). Local differences in exposure relating to these inputs may lead to notable differences in microbiota composition between different flocks, even for housing systems of the same type (Cressman *et al*., 2010; Park *et al*., 2016; Richards *et al*., 2019). This is likely to confound any interpretation in the context of differing welfare between alternative forms of housing system in the present study. Indeed, despite their almost identical rearing conditions from one-day-old, large differences in relative ASV abundances were detected between the sampled H&N and Hyline pullets ($R^2 = 0.316$). Microbes inhabiting the gut are derived from the environment during incubation, hatching and handling during delivery, and this variable colonization has been found to lead to marked differences in the composition of the initial intestinal microbiota between young chicks from different hatcheries, or different buildings within a hatchery (Pedroso *et al*., 2005; Richards *et al*., 2019). Though potential effects of genotype should not be discounted, it has been concluded that environmental exposure, diet and management practices are more influential than genetics in shaping the caecal microbiota (Pedroso *et al*., 2005; Richards *et al*., 2019). As the pullets in the present study originated from different hatcheries and were raised in separate barns, the differences between them are likely to be predominantly environmental in origin. On the other hand, hens (of a single strain) within a housing system/flock generally originate from the same hatchery and are subsequently exposed to collective microbial environments during rearing and adult housing, as was the case with the sampled adult birds. Therefore, although likely smaller in magnitude, individual differences in microbiota composition existing within a flock may reflect experiential or intrinsic factors with implications for welfare, such as metabolic efficiency/feed conversion ratio (Singh *et al*., 2012). For these reasons, further comparison of caecal microbiome compositions was limited to the analysis of within-system differences, in relation to physical body condition.

Initially, Gneiss analysis was employed to compare caecal ASV abundance between samples from hens with good and poor body conditions from the multi-tier housing system (1,188 ASVs). The covariate physical body condition was found to account for 13% of the observed variance (R^2 = 0.13). Differentially abundant ASVs in samples from the multi-tier hens are displayed in Table 5.3, classified at the taxonomic level of family.

Table 5.3. Families of caecal microbiota containing amplicon sequence variants (ASVs) identified as differentially abundant between adult hens with good and poor physical body conditions, and which differed significantly from an expected distribution (displayed in brackets).

		Number of ASVs			Chi-Square Test	
Family	Total	> Abundance in Good Condition Hens	> Abundance in Poor Condition Hens	Not Differentially Abundant	Test Statistic (χ^2)	p value
Ruminococcacceae	285	42 (27)	85 (83)	158 (174)	9.43	0.009
Clostridiales vadin BB60 group	87	6 (8)	16 (25)	65 (53)	6.74	0.03
Bacteriodaceae	70	13 (7)	18 (20)	39 (43)	6.51	0.04
Veillonellaceae	23	0 (2)	12 (7)	11 (14)	7.03	0.03
Methanomethylophilaceae	9	0 (1)	6 (3)	3 (6)	6.33	0.04
Puniceicoccaceae	6	0 (1)	5 (2)	1 (4)	8.54	0.01

In the enriched cage system, the overall linear regression model fit was R^2 = 0.049, with covariate physical body condition accounting for 4.9% of variance. The smaller degree of variation relating to body condition in this (indoor only) system may be explicable in terms of differences in ranging behaviour between the body condition groups from the multi-tier system, wherein good condition hens were sampled directly from the outdoor range, while poor condition hens may not have had direct contact with soil. At the level of order, no ASVs were differentially abundant between the two physical condition groups from the enriched cage system. Whether the families that exhibited differing relative abundance in the multi-tier system (Table 5.2) were also differentially abundant in the enriched cage system was therefore investigated. Only two families displayed significantly different abundance between enriched-caged hens of good and poor physical condition. The first of these was *Puniceicoccaceae,* a poorly characterised and largely environmental group that was present only at a very low relative abundance. The second family was *Methanomethylophilaceae*, a group of methanogenic archea. ASVs assigned to *Methanomethylophilaceae* had a significantly higher relative abundance in samples from hens in poor physical condition than from hens in good physical condition ($\chi^2_1 = 6.33$, $p = 0.04$), with nearly all relative abundance contributed by ASVs that were more abundant in poor condition hens. Specifically, of nine *Methanomethylophilaceae* ASVs detected, six were more abundant in poor condition birds, while three were not differentially abundant between good and poor condition birds.

Approximate relative proportions of *Methanomethylophilaceae* ASVs were 1% for poor condition hens compared to 0.1% for good condition hens in the multi-tier (free range) system, and 0.2% for poor condition hens compared to < 0.1 or < 0.01% for good condition hens in the enriched cages.

5.5 Discussion

The present study explored whether two common types of commercial housing system were associated with differing degrees of chronic stressful experience for laying hens, as reflected by levels of adult neurogenesis in the HF. To assess within-system variation in cumulative experience, individuals of good and poor physical condition from each system were additionally compared. Densities of multipolar differentiating immature neurons expressing DCX were lower in hens of poor condition from both the multi-tier aviary system (with outdoor range) and the enriched cage system. There was no main effect of housing system on this measure of AHN, suggesting that neither alternative was associated with an overall shared experience that was more stressful (for all of the hens) than the other. As the multi-tier aviary housed H&N hens, while the enriched cage system housed Hy-Line birds, a sample of 14-week-old pullets of each strain was also collected directly from the rearing farm. No baseline strain difference in AHN was apparent prior to transfer to adult housing, making it unlikely that a genetically-determined strain difference in initial levels of AHN obscured an effect of differing shared experience in adult housing when measured towards the end of lay. However, strain-specific differences in stress resilience, or in age-dependent changes in AHN, cannot be ruled out.

5.5.1 AHN did not differ between hens housed in a multi-tier aviary and an enriched cage system

Given the differences between multi-tier aviary system and enriched colony cage system sampled, it is perhaps surprising that no group difference in AHN was observed following long-term experience of these conditions. Group sizes were ~4,000 in the former compared to 50 in the latter, while hens in the multi-tier system had the option of visiting the outdoor range. While the colony system offered forms of enrichment, such as the scratch matt with grit auger to stimulate foraging, the multi-tier system constituted a larger and generally more complex space. Given that AHN is stimulated by enriched environments in rats and mice (e.g. Van Praag *et al.*, 1999; Kronenberg *et al.*, 2003) and spatial memory challenge in birds (Patel *et al.*, 1997; LaDage *et al.*, 2010), it is perhaps surprising that DCX^+ differentiating cell densities were not higher in the multi-tier aviary. Though internal stocking densities were not dramatically different in the two systems, freedom of movement (especially vertical movement) is also

greater in multi-tier systems than enriched cages (Lay Jr. *et al.*, 2011), and birds in the former housing are more active (Rodenburg *et al.*, 2008). While running exercise upregulates AHN in rats and mice (e.g. Van Praag *et al*., 1999), the stimulatory effect of flight tunnel exercise on AHN in European starlings has been found to depend upon dietary levels of vitamin E (Hall *et al*., 2014). Further investigation of the nature of the exercise-neurogenesis association in birds, and specifically in chickens, may therefore be required. Generally, at least in rodents, modulation of AHN levels integrates positive and negative experiences occurring simultaneously or sequentially (e.g. Fabel *et al.*, 2009; Veena *et al.*, 2009; Kiuchi *et al.*, 2012; Kim *et al.*, 2013). Comparable levels of AHN at the end of lay may therefore reflect a collation of the various factors in each system that have a positive and negative impact on cumulative experience.

Effects of outdoor access are generally considered positive (Norwood and Lusk, 2011), but many birds do not regularly go outside (Richards *et al.*, 2011), and those in the worst condition might choose to remain closest to resources in the barn. Tracking the movement of hens has revealed that those with severe keel bone fractures spend more time on the top tier of a multi-tier aviary, and less time in the lower tiers and litter (Rufener *et al.*, 2019). In the same study, time spent on the top tier also increased with advancing age. Food, water and nest boxes are all provided on the top tier, while the elevated roosting positions offered may be favoured by instinctive anti-predatory behaviour (Newberry *et al.*, 2001). Animal density during light hours (Channing *et al.*, 2001) and agonistic interactions (Hansen, 1994), which some hens may wish to avoid, are also more frequent in lower tiers (Rufener *et al.*, 2019). It is therefore possible that hens in the generally poorest physical condition, such as the subgroup sampled from the multi-tier system, may spend the majority of their time on the upper tiers and consequently not benefit from outdoor access. The increased prevalence of keel bone fractures is another notable negative consequence of cage-free systems (Rodenburg *et al.*, 2008; Lay Jr. *et al.*, 2011), but birds exhibiting such damage were excluded from the present sample due to thorough exploration of this welfare issue in Chapter 4. Though not true for the flocks sampled here, mortality is generally found to be higher in litter-based housing systems than in furnished cages (Michel and Huonnic, 2003; Rodenburg *et al.*, 2008). This observation may relate to higher airborne concentrations of dust and bacteria (Rodenburg *et al.*, 2008) and a greater prevalence of bacterial and parasitic diseases in non-cage (litter-based and free-range) housing systems (Mazaheri *et al.*, 2005; Fossum *et al.*, 2009). Exposure to soil in free-range systems further increases contact with infectious microorganisms, and these can also be transferred from wild animals (reviewed in Lay Jr. *et al.*, 2011). The incidence of cannibalism (i.e. feather pecking

leading to mortality) is also higher in non-cage housing systems than furnished cages (Fossum *et al.*, 2009), while other undesirable behaviours, such as piling and smothering, may also be more common, and more difficult to control (Bright and Johnson, 2011; Lay Jr. *et al.*, 2011).

Group size differed dramatically between the housing systems compared, equating to 50 individuals in the enriched cages and 4,000 individuals in the multi-tier system. While group size is often perceived to be a likely important influence on hen welfare, it is not clear what social system hens would prefer (Dawkins, 1982; Weeks and Nicol, 2006). The proclivity of hens in small groups to form stable, linear dominance hierarchies is well documented (e.g. Rushen, 1982), and flocks of red junglefowl and feral domestic fowl typically range in size from five to 48 individuals (Nicol *et al.*, 1999). Hens reliably choose to associate with familiar hens over unfamiliar hens (Dawkins, 1982; Bradshaw, 1992a; Dawkins, 1995). Moreover, encounters with unfamiliar birds are stressful, leading to acute increases in heart rate (Candland *et al.*, 1969), and to lower weight gain and higher plasma CORT (Gross and Siegel, 1981), feed intake, abdominal fat, and H:L ratios (Anthony *et al.*, 1988) when repeated on a longer-term basis. Introduction of unfamiliar birds into a group results in increased aggression (Craig *et al.*, 1969), and aggression towards subordinate birds may be associated with group size (Gibson *et al.*, 1988). It is thought that the ability of hens to recognize other individuals may be limited to groups of around 80 (Appleby *et al.*, 1985; Nicol *et al.*, 1999), as hens in a flock of 96 formed an incomplete dominance hierarchy (Guhl, 1953). Hens have been shown to prefer to be in a group of 70 than a group of four, both when stocking densities were constant and when densities were higher in the larger group (Lindberg and Nicol, 1996). In line with apparent constraints on the ability of hens to recognize others from long distances, it was thought that unnaturally large commercial flocks, where hens repeatedly encountered unfamiliar individuals at ranges close enough to induce agonistic encounters, may have negative implications for welfare (Dawkins, 1995). It has been suggested that failure to recognize individual hens in cage-free systems with large flocks might thus lead to increased levels of aggression, particularly impacting low-ranked birds (Downing and Bryden, 2002). However, aggressive behaviour has been reported to be less frequent in flocks of 300 birds than in small to medium-sized flocks (Hughes *et al.*, 1997; Weeks and Nicol, 2006), as hens may switch to a strategy of non-recognition whereby they do not attempt to form social relationships (Hughes *et al.*, 1997; Nicol *et al.*, 1999). In this scenario, aggression may instead arise from comparison of body and comb sizes during direct assessments (Weeks and Nicol, 2006). Which housing system had the group size likely to be associated with the greater overall stressful experience is therefore unclear.

Previous reviews have concluded that no single housing system is ideal from the perspective of welfare, as each is associated with unique limitations (Rodenburg *et al.*, 2008; Lay Jr. *et al.*, 2011). In an exploration of the consequences of housing system for forebrain morphology, Patzke *et al.* (2009) compared hens housed in battery cages, small littered ground pens and a free range system. While these conditions are arguably more extreme in their differences than the commercial housing alternatives compared in the present study, the authors concluded that observed differences in cell body sizes and serotonergic and catecholaminergic innervation patterns in the HF and nidopallium caudolaterale were surprisingly mild. This was interpreted as reflecting limited sensitivity of the adult laying hen brain to the environment. However, a previous study that compared six flocks from furnished cages and seven from non-cage systems found no significant difference in an integrated welfare score, which collated multiple indicators including fearfulness, plumage, body and bone condition, dust levels and mortality (Rodenburg *et al.*, 2008). Moreover, Patzke *et al.* (2009) did observe an altered serotonergic innervation pattern in the free range hens, which was attributed to this group displaying the highest incidence of feather pecking and poorest plumage condition. In all flocks, some individuals are more afflicted by pecking from conspecifics than others (e.g. Lindberg and Nicol, 1996; Bilcik and Keeling, 1999). Collectively, these findings support the present interpretation that a trade-off between positive and negative experiences in each system (e.g. greater complexity but more deleterious behaviour such as feather pecking in the free range system) may result in little net difference in hippocampal plasticity, but that AHN is sensitive to a higher degree of stress experienced by individuals with the poorest welfare.

It may also be that the sample sizes employed were not sufficient to reflect an inter-system effect, due to a high level of intra-system variability. Hens were selected in a manner intended to be representative of the entirety of each housing system, for example by (to the extent possible) sampling each cage position in the enriched cage system. There is evidence that cage position is associated with varying levels of stress, with higher fearfulness and mortality, and lower indices of production, observed in hens from upper cage-tiers (Sefton, 1976; Jones, 1985; Jackson and Waldroup, 1987; Hemsworth and Barnett, 1989). This effect may stem from long-term differences in light intensity and exposure to visual stimuli (Jackson and Waldroup, 1987). When sampling from the multi-tier system, the decision was made to conflate good physical condition with apparent use of the range, according to which hens ventured outside on the day of sampling. As individual variation in ranging behaviour was not measured, but may be substantial (e.g. Richards *et al.*, 2011; Gebhardt-Henrich *et al.*, 2014), the individual good condition hens selected from the multi-tier system may have experienced different habitual

levels of spatial-cognitive stimulation and exercise, which might relate to AHN on a linear basis (see Chapter 3) that could also obscure group differences. It should also be noted that the degree of chronic stress required to first induce a detectable decrease in AHN could be relatively high. This means that differences in welfare (i.e. overall stressful experience) may still have existed between hens from the two types of commercial housing system, but these did not reach the threshold detectable through quantification of immature neurons. It can nevertheless be concluded that these differences in experience must have been smaller in magnitude than the differences between good and poor condition hens in the same flock.

5.5.2 Lower AHN was observed in hens in poor physical condition

While AHN did not differ between adult hens housed in a multi-tier free range and a colony enriched cage housing system, levels of DCX^+ immature neurons over the whole HF were lower in individual hens with low body mass, poor feather cover and pale combs than in individuals with the opposite characteristics. This suggests that variation in experience within commercial housing systems may exceed overall differences in shared experience between caged and non-caged systems. As several factors may contribute to poor physical condition, these same factors may also influence the cumulative experience of stress. Other measures taken from the sampled hens may indicate which general factors or systems are likely to be involved.

Firstly, hens in poor physical condition from both housing systems exhibited a higher abundance of methanogenic archea (*Methanomethylophilaceae*) in the caecum. These anaerobic archea inhabit the gastrointestinal tract (Saengkerdsub *et al.*, 2007) and reduce carbon dioxide, formic acid, or methylamines to methane (CH_4), resulting in a loss of feed energy (Patra *et al.*, 2017). Given that this difference was also present in the enriched-cage hens, it is unlikely to be attributable purely to individual differences in going outside and contact with soil, as could have been the case in the free range system. Recent work has recognized the gut microbiota as key to the regulation of immune function, metabolism, and welfare (Hubert *et al.*, 2019), though studies to date do not appear to have specifically explored the implications of methanogen abundance for welfare. Generally, however, complex bidirectional interactions exist between the gut microbiota, central nervous system and immune system (reviewed in Petra *et al.*, 2015). In relation to stress, activation of the hypothalamic-pituitary-adrenal (HPA) axis regulates immune cells and alters permeability in the gut, which subsequently affects composition of the microbiome (Dinan and Cryan, 2012; Petra *et al.*, 2015). Both physiological and emotional stress have been demonstrated to influence the composition of gut microbiota (Dinan and Cryan, 2012). For example, exposure to chronic social stress decreased the relative

abundance of *Bacteroides* species and increased the abundance of *Clostridium* species in the caecum of adult mice, as well as activating the immune system (Bailey *et al.*, 2011). Just as the HPA-axis affects microbiome composition, evidence also suggests that microbiota influence functioning of the HPA-axis. Challenge with pathogen *Citrobacter rodentium* significantly increased anxiety-like behavior in mice measured seven to eight hours later, as evidenced by increased avoidance of the centre area of an arena and risk assessment behavior (Lyte *et al.*, 2006). C-fos expression was also increased in vagal sensory ganglia, consistent with the transmission of mediating signals from the gut to the brain. On the other hand, mice raised in a germ-free environment have been observed to exhibit decreased anxiety behaviour in the elevated plus maze and the light/dark box tests (Heijtz *et al.*, 2011). Germ-free mice were also found to secrete substantially higher levels of plasma ACTH and CORT in response to restraint stress, and this difference from controls was reversed by colonisation with *Bifidobacterium infantis*, but further enhanced by mono-association with pathogenic *Escherichia coli* (Sudo et al., 2004). These studies suggest that microbiota may have a role in programming responsivity of the HPA-axis (Dinan and Cryan, 2012). The notion that the microbiome might also influence AHN is supported by the finding that long-term (seven-week) treatment with broad-spectrum antibiotics that severely depleted the intestinal microbiota of mice also decreased the number of $BrdU^+$ cells (co-expressing both DCX & NeuN and NeuN alone) in the dentate gyrus (Möhle *et al.*, 2016). As such, the finding that poor condition hens have suppressed AHN and an altered microbiome composition may be explicable in terms of interactions between stress and immune function, which future studies may seek to disentangle.

Ill health may present a cause of stress and poor physical condition in laying hens. A critical brain-mediated response to immune challenge is activation of the HPA-axis, to mobilise energy stores and modulate immune responses (Zimomra *et al.*, 2011). Infective agents (e.g. gut pathogens, such as *Escherichia coli*) therefore lead to a rise in circulating CORT (Dinan and Cryan, 2012). While this acute stress response augments immunity, long-term stress is immunosuppressive (Dhabhar, 2009). Moreover, research in mice indicates that CORT is causally involved in reductions in AHN that occur in the context of chronic negative experience (Lehmann *et al.*, 2013). Repeated or prolonged activation of the HPA-axis by recurrent infections or other sources of chronic ill health may therefore have detrimental consequences for neurogenesis. However, though AHN is known to be impaired by several neuropathic viruses (e.g. Herpes simplex virus type-1, coxsackievirus, Borna disease virus, human immunodeficiency virus, cytomegalovirus, Zika virus) (Li Puma *et al.*, 2019), the association

between infections that do not directly affect the brain and survival of immature neurons in the HF does not yet appear to have received much attention.

Interestingly, expression of mRNA for the pro-inflammatory cytokine interleukin-6 (*IL-6*) was lower in poor condition hens when compared to their better condition flock-mates. IL-6 is one of the primary cytokines driving HPA-axis activation in response to immune challenge (Zimomra *et al.*, 2011), and previous research suggests that differences in its expression may reflect efficiency of the immune response. Certain inbred lines of chickens are characterized by either resistance or susceptibility to systemic salmonellosis, and white blood cells (macrophages) from resistant lines clear this bacteria within 24 to 48 hours of infection, while Salmonella persists in the cells of susceptible lines (Wigley *et al.*, 2002). Greater expression of *IL-6* has been demonstrated by both macrophages and heterophils (two forms of phagocyte white blood cell that can be isolated from the spleen (Sekelova *et al.*, 2017)) of hens from the Salmonella-resistant lines compared to the susceptible lines, and is thought to be associated with more efficient initiation of innate and adaptive immune responses (Swaggerty *et al.*, 2004; Wigley *et al.*, 2006). In an outbred chicken line (the Rhode Island Red), responses of macrophages were variable, with individual birds exhibiting either a high response comparable to resistant line cells, or a low response similar to that of susceptible-line cells (Wigley *et al.*, 2002). In the case of commercial hybrid strains, such as Hy-Line and H&N brown layers, an intermediate level of individual variability in macrophage efficacy might be expected. As such, the observed differences in expression of *IL-6* within hens of both strains may reflect variation in systemic innate immunity, thought to play a major role in susceptibility to infection (reviewed in Wigley *et al.*, 2006). Though chronic elevation of pro-inflammatory cytokine expression may be associated with inflammation and poor welfare (Weber *et al.*, 2016), commercially-housed hens are exposed to many species of pathogen (Fossum *et al.*, 2009), and a greater magnitude of acute pro-inflammatory response to immune challenge may contribute to maintenance of good physical condition. While differences in innate immunity may be genetic, chronic stress of other origins also suppresses or dysregulates immune function and increases susceptibility to disease (Dhabhar, 2009), for example as evidenced by inhibited antibody production (El-Lethey *et al.*, 2000; El-Lethey *et al.*, 2003; Mashaly *et al.*, 2004). The design of the present study does not allow us to ascertain whether poor innate immunity led to ill health, which constituted the stressor that downregulated AHN, or whether chronic stress arising from other sources concurrently downregulated AHN and caused immunosuppression, increasing susceptibility to ill health.

Studies in chickens have indicated that the mass of the spleen is reduced by chronic administration of CORT (Puvadolpirod and Thaxton, 2000; Iyasere *et al.*, 2017) or upstream mediator ACTH (Davison *et al.*, 1985), or by exposure to unpredictable chronic mild stress (Gualtieri *et al.*, 2019). In terms of positive experience, mice housed in enriched conditions exhibit larger spleens than standard-housed mice (Gurfein *et al.*, 2012; Gurfein *et al.*, 2014). It is thus perhaps surprising that poor condition birds from the enriched cage system had larger spleens that the other subgroups. However, there is an avian Hepevirus that causes a condition known as big liver and spleen (BLS) disease, which produces enlarged spleens weighing over one gram per kilogram of body mass (Clarke *et al.*, 1990; ThePoultrySite, n.d.). A ratio of 1.28g spleen mass per kilogram of body mass was observed in the sampled enriched cage/poor condition hens, compared to ratios of 1.01g, 0.99g, and 0.87g per kilogram in the enriched cage/good condition, multi-tier/good condition and multi-tier/poor condition groups, respectively. These observations suggest that the poor condition hens from the enriched cage system may have suffered from such an infection, though this could not be conclusively determined without a specific test. Liver samples were unfortunately not weighed or collected in the present study.

At least in group sizes for which hens are able to recognize other individuals, aggressive pecks are used maintain the dominance hierarchy (Rushen, 1982). The number of pecks received correlates negatively with feather cover and body size (Bilcik and Keeling, 1999), while hens with paler combs may be more likely to be subordinate (Bradshaw, 1992b; Cloutier *et al.*, 1996). The relationship between social status and AHN has yet to be explored in chickens, but in mountain chickadees (*Poecile gambeli*), subordinate individuals have a lower level of proliferation in the hippocampal ventricular zone (Pravosudov and Omanska, 2005), suggesting the avian brain may be sensitive to stress arising from low social status. Subordinate status therefore represents another factor which might contribute to greater cumulative stressful experience for hens in a poorer physical condition. As the "poor condition" hens selected were characterized by low body weight, it is possible that inadequate nutrition may have led both to this outcome and to limitations in AHN. However, while poor health might be associated with stress, and could also interact with feed consumption or efficiency, several studies in rats and mice have demonstrated that dietary restriction alone actually upregulates levels of AHN (e.g. Lee *et al.*, 2000; Lee *et al.*, 2002; Kitamura *et al.*, 2006). It therefore seems unlikely that the association between physical condition and AHN can be attributed solely to nutrition. Exercise robustly upregulates AHN in the HF of rats and mice (e.g. Van Praag *et al.*, 1999; Snyder *et al.*, 2009), but given that the finding of relatively greater levels of AHN in good condition birds

was not limited to the free range system, where these individuals went outside, this factor was probably not a significant contributor to the quantified levels of AHN. If movement had a major impact on AHN at the end of lay, numbers of DCX^+ cells would be predicted to be generally higher in hens from the multi-tier system.

As this study did not involve experimental manipulation, the direction of cause and effect cannot be equivocally determined. It is therefore also possible that the influence of existing individual differences in AHN on personality and behaviour manifested in differences in external physical condition between hens. Future controlled experiments will be necessary to conclusively determine which factors contribute causally to the differential experience of chronic stress for commercial laying hens.

5.5.3 Conclusions

Overall, a group difference in levels of AHN towards the end of lay was not observed between hens housed in a multi-tier system with an outdoor range and an enriched cage system. As a combination of factors with positive and negative implications for welfare are associated with each system, it may be that these exert opposing influences on AHN, equating to little difference in the overall magnitude of stressful experience. It is also possible that differences between the two systems were obscured by a high level of variability within each system, as individuals in poor physical condition had lower levels of DCX^+ immature neurons than individuals in relatively good physical condition. Physical health may be associated with overall stressful experience, as hens in poor condition had lower expression of pro-inflammatory cytokine *IL-6* in the spleen, a higher abundance of methanogenic bacteria in the caecum, and in the case of the enriched cage system, enlarged spleens. Social stress relating to subordinate status may also contribute to chronic stress. These findings suggest that housing systems which produce the lowest proportion of birds in a poor physical condition may be associated with the "best" relative overall welfare. Future systematic sampling studies might undertake this comparison.

5.6 References

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Chapter 6. General Discussion

6.1 Summary of Research

The reported research project explored the association of adult neurogenesis in the laying hen HF with various commercially relevant long-term experiences and conditions, to assess which of these are likely to have the greatest impact on overall welfare, and whether adult hippocampal neurogenesis (AHN) may comprise a biomarker of long-term experience with greater sensitivity than existing measures. Results from four studies offer several interesting insights into factors with apparent influence for the cumulative stressful experience of adult hens. Specifically, a relative suppression of numbers of differentiating immature neurons in the HF indicated that both severe keel bone fractures (KBFs) and poor physical condition, possibly associated with ill health, were of sufficient salience and magnitude to induce a state of chronic stress in affected hens. While a within-bird comparison suggested that transcription of the *DCX* gene might constitute a poor quantitative proxy for numbers of immature neurons, hippocampal expression of proliferative marker *PCNA* may also be associated with individual differences in environmental complexity, afforded by use of outdoor areas, and in behavioural strategy (or coping style). The findings also contribute to the understanding of functional homologies between neurogenesis in the adult avian and mammalian HFs, and highlight methodological considerations for related future research.

6.2 Alternative Methods of Quantifying AHN

Traditional morphological methods of quantifying numbers of immature neurons produced through AHN (i.e. immunohistochemistry (IHC) and counting cells in serial sections) are generally relatively time-consuming. Previously, transcription of the *DCX* gene in the dentate gyrus (DG) was demonstrated to reflect group differences in the DCX-immunoreactive cell densities of mice kept in control and enriched housing conditions (Gualtieri *et al.*, 2017), suggesting that mRNA levels may comprise a proxy biomarker capable of differentiating between the long-term experience of groups of animals. The initial studies conducted for this research project (reported in Chapters 2 & 3) therefore explored whether use of real-time quantitative PCR (qPCR) to measure mRNA for *DCX* and related candidate genes would reflect differential experience, relating to experimental housing conditions and use of outdoor areas, respectively. Whilst neither expression of *DCX* mRNA, nor of mRNA for proliferating cell nuclear antigen (*PCNA*) or the ratio of mineralocorticoid (*MR*) to glucocorticoid (*GR*) receptors, differed between the two housing conditions, the same was true for existing measures of welfare

(serum CORT, H:L ratio, etc.). It was thus unclear whether transcription lacked sensitivity as a quantitative proxy, or whether the cumulative experience associated with the alternative housing conditions designed was not sufficiently different to induce alterations in AHN and glucocorticoid regulation. Chapter 3 sought to relate individual differences in time spent in outdoor areas of a free-range system, and in the tonic immobility response, to expression of mRNA for *DCX* and *PCNA*. While time spent in uncovered areas (the stone yard and range) positively predicted *PCNA* expression in the rostral (septal) HF, consistent with hypothesized subregional specialization in the avian HF (Smulders, 2017), *DCX* expression did not display the same pattern. According to current understanding of the role of adult-born cells, functional explanations that relate increased levels of proliferation to the cognitive stimulation experienced while ranging, and to enhanced behavioural plasticity associated with a reactive coping style, depend upon the translation of upregulated proliferation in the HF to a greater proportion of surviving immature neurons. However, it remained possible that stress arising from certain aspects of ranging outside preferentially reduced numbers of fate-determined neuronal progenitors, without affecting proliferation.

In Chapter 4, a within-bird methodological comparison was conducted, with tissue from one hemisphere utilized for DCX IHC, while qPCR was employed to quantify *DCX* mRNA in the HF of the remaining hemisphere. Where DCX^+ stained cell densities exhibited a strong suppressive effect of severe KBFs on AHN, this group difference was not reflected by the expression of *DCX* mRNA. On an individual level, molar quantities of *DCX* mRNA also did not correlate with the density of DCX^+ cells in the HF. The results of this direct comparison suggests that transcription of the *DCX* gene presents a poor proxy for neuronal differentiation in the chicken HF. Lateralization of particular spatial-cognitive functions in the homing pigeon HF is well-documented (Bingman *et al.*, 2006; Jonckers *et al.*, 2015), and HF lesion studies point to certain lateralized cognitive mechanisms in domestic chicks (Tommasi *et al.*, 2003). Differences in actual levels of AHN between the hemispheres could therefore have contributed to a lack of within-subject correlation. Rather than confounding method and hemisphere, half of the samples from the left hemisphere of hens with minimal KBFs were used for IHC, while the other half were used for qPCR. The remaining samples from the right hemisphere were allocated to the opposite techniques, and tissue from hens in the severe KBF group was used in a similarly balanced manner. When results from the two hemispheres are compared between the samples quantified in the same manner (i.e. half from the left and half from the right hemisphere for each technique), neither DCX^+ cell densities nor *DCX* mRNA expression (corrected for *LBR*) differ between the left and right HF (multipolar DCX^+ cell density: $F_{1,17.0}$

= 0.18, p = 0.675; bipolar DCX$^+$ cell density: $F_{1,17.1}$ = 3.05, p = 0.099; *DCX* mRNA: $F_{1,19.5}$ = 0.50, p = 0.487). This suggests that the observed discrepancy is instead methodological in origin.

Generally, reviews have concluded that only weak correlations exist between mRNA transcript and protein abundances in many scenarios (Maier *et al.*, 2009; Liu *et al.*, 2016), as variation is introduced by multiple biological and technical parameters. Biological factors identified include temporal delays between transcription and protein synthesis, effects of the specific genetic sequence and the binding of regulatory elements on translation, the local availability of ribosomes, polymerases and other resources for protein synthesis, and modulations of protein half-life occurring independent of transcription (Liu *et al.*, 2016). Additional sources of variation arise when attempting to relate mRNA abundance to numbers of stained cell bodies expressing the protein. For example, individual cells vary in their overall transcription, in a manner which correlates with cell volume (Padovan-Merhar *et al.*, 2015). *DCX* mRNA abundance was closest to being correlated with the density of multipolar DCX$^+$ cells, compared to bipolar cells alone or multipolar and bipolar cells combined (see section 4.4.6), which may suggest that soma size was indeed an influential determinant of relative mRNA levels. Protein synthesis may also be temporally dynamic throughout the process of neuronal migration and differentiation.

In the mouse DG, baseline patterns of DCX expression across the septo-temporal axis differed when measured in terms of *DCX* mRNA transcripts or immunoreactive cells stained via IHC (Gualtieri *et al.*, 2017). However, running exercise has been noted to induce the same changes in *DCX* mRNA and IHC quantification (Kremer *et al.*, 2013; Gualtieri *et al.*, 2017). This is consistent with the finding that differentially expressed mRNAs in mouse tissue correlate significantly better with their protein product than non-differentially expressed mRNAs (Koussounadis *et al.*, 2015). Though differential densities of DCX$^+$ cells with KBF severity were observed in the laying hen HF using IHC, this was not reflected by the abundance of *DCX* mRNA in hippocampal tissue. This may be a consequence of the more limited number of technical control measures currently applicable to AHN in the avian HF, compared to in rats and mice. Given that AHN in the mammalian brain is restricted to the DG substructure, this region was micro-dissected from the larger hippocampus for the analyses conducted in Gualtieri *et al.* (2017). Moreover, AHN in mammals is specific to the population of granule cells within the DG, which are unique in their expression of genetic marker *Prox1* during adulthood (Karalay *et al.*, 2011; Stergiopoulos *et al.*, 2014). It is therefore possible to employ *Prox1* as a housekeeping gene, in order to normalize expression of *DCX* within the homogenized tissue

with respect to the total volume of granule cells present therein. This acts as a control for specificity of the dissection technique, accounting for inclusion of any tissue not belonging to the DG. Compared to the discrete neurogenic cell population of mammalian granule cells, a wider range of cell types are added to the existing circuity of the avian brain during adulthood, meaning that an equivalent normalization technique is not possible. To normalize expression relative to the total volume of mature neurons sampled, use of mature neuronal marker NeuN as a control gene was explored, but this did not improve the sensitivity afforded by transcription of the *DCX* gene to discriminate between groups of hens. This is likely because any telencephalic tissue adjacent to the HF that was accidentally included in the samples dissected would have also included mature neurons.

Previous research suggests that the relative unreliability of *DCX* mRNA transcript abundance as a proxy for densities of differentiating neurons might be attributable to low-level background expression of this gene. Background expression of *DCX* mRNA has been observed in non-neurogenic subdivisions of the mouse hippocampus (i.e. beyond the DG), and transcript levels in these regions proved unresponsive to running exercise (Kremer *et al.*, 2013). It has been suggested that fully differentiated, adult neurons may have the capacity to express DCX during changes in axon or dendrite growth, or synaptogenesis (Nacher *et al.*, 2001). While this low-level expression in mature cells should not result in staining of the cell body during IHC, it may be reflected in transcript abundance in homogenised tissue. Though the majority of the avian telencephalon is neurogenic, low-level transcription of *DCX* in other types of HF cell may obscure correlations with expression of this marker by differentiating immature neurons. A similar mechanism of background expression in the avian brain is suggested by the observation of some weakly labelled DCX-immunoreactive structures in various di- and mesencephalic nuclei of the adult canary brain (Boseret *et al.*, 2007; Balthazart *et al.*, 2008). It was concluded that this weak immunoreactive signal was not associated with AHN, and was presumably related to cellular plasticity of mature adult neurons, in terms of reorganizations of the dendritic arbor (Nacher *et al.*, 2001; Brown *et al.*, 2003). As such, background *DCX* mRNA expression in non-neurogenic cells (e.g. mature neurons during dendritic remodeling) within the chicken HF, which is insensitive to experiential factors, may preclude a relationship with DCX IHC - even if the mRNA transcript is differentially expressed by immature neurons.

This issue of background expression may be specific to the *DCX* gene. For example, while patterns across the septo-temporal DG axis in control mice differed between DCX mRNA and IHC, the pattern for neurotrophic factor BDNF indicated by the two methods was the same (Gualtieri *et al.*, 2017). This could therefore explain the discrepant relationships that expression

of mRNA for *PCNA* and *DCX* displayed with tonic immobility and time spent in outdoor areas in Chapter 3. PCNA comprises a non-specific marker which is expressed by dividing cells with various eventual fates, in a manner dependent upon cell-cycle phase (Kurki *et al.*, 1986). In the adult rat DG, around 80% of surviving proliferating cells become neurons, whereas ~15% become astroglia (Lee *et al.*, 2006). Therefore, while PCNA is not a marker of AHN *per se*, its expression in the HF is likely to predominantly reflect the initial stage of the production of new neurons. Members of our research group have previously localized $PCNA^{+}$ cells to the HF border of the ventricular zone in the chicken brain (unpublished data), as has been demonstrated in pigeons (Mazengenya *et al*., 2017).

Due to the issue of background expression, it is possible that other molecular markers of neuronal-fate-determined stages of AHN offer greater reliability than *DCX*. Potential candidates include the pro-neuronal gene neurogenic differentiation 1 (NeuroD1), which is expressed in type-2b and -3 progenitor cells (Gao *et al.*, 2009), and the calcium-binding protein calretinin, which is transiently expressed during the early post-mitotic phase (Brandt *et al.*, 2003). However, in the rodent brain, calretinin is also expressed in mature interneurons and mossy cells, and this lack of specificity means that Prox-1 co-expression is often required as a control for the immature neuronal phenotype (Tanti *et al.*, 2013). Future work may explore the relative sensitivity of transcript expression for alternative markers of AHN to experience.

Overall, it is perhaps reasonable to conclude that the results regarding *DCX* mRNA expression in Chapters 2 and 3 should be viewed with some skepticism. While it is true that experiences associated with ranging outside, such as social isolation, sightings of predators and exposure to the elements, may indeed be stressful for hens, the observed negative correlation between ranging and *DCX* mRNA expression in the caudal HF must be verified through the morphological quantification of immature neurons.

6.3 Factors Associated with Differences in Long-Term Experience for Hens

Over the course of four studies, comparison of novel and existing measures of long-term experience in laying hens was undertaken in relation to the use of external areas, severity of naturally occurring KBFs, activity within a multi-tier aviary, physical body condition, and differing experimental and commercial housing conditions. Several conclusions regarding the implications of these various factors for long-term welfare can be drawn.

6.3.1 Physical health, inflammation & chronic stress

Previously, Patzke *et al.* (2009) concluded that plasticity in the forebrain of adult hens exhibited little sensitivity to environmental conditions. However, in the present research, reduced levels of AHN in laying hens were observed in association with severe KBFs and generally poor body condition, reflected by low body mass, poor feather cover and a pale comb. KBFs are likely to be the cause of chronic pain and (physiological) stress, rather than vice versa, but for the latter birds it is unclear whether psychological stress or illness constituted the primary catalyst leading to the eventual suppression of AHN. While illness itself is a stressor, other forms of stress also lead to an increased incidence of parasitic diseases in domestic livestock (Broom, 2001). The initial causal factor may even have differed for individual sampled hens. However, as a group, the poor condition hens were also characterised by altered splenic *IL-6* expression and compositions of the caecal microbiome. These observations suggest that the pathways relating both severe KBFs and poor physical condition to downregulated AHN may be similarly mediated by mechanisms relating HPA-activation to inflammation.

While the commercial hens sampled in Chapter 5 experienced the same housing conditions as their system- or flock-mates, only a subset of these exhibited a poor body condition. Low body weight and poor plumage (two of the criteria employed) have previously been linked to stress and activation of the HPA-axis in laying hens (Campo *et al.*, 2001; El-lethey *et al.*, 2001), while evidence suggests that poor body condition is associated with ill health. Body condition score is an accurate indicator of health status in mice, proving more informative than body weight alone (Ullman-Culleré and Foltz, 1999), and is associated with multiple health parameters in dairy cows (Markusfeld *et al.*, 1997; Roche *et al.*, 2009). Hens lived in close contact with the (50) individuals in their furnished cage, or at least a proportion of the (4,000) birds in their multi-tier flock. It is usually easy for pathogens to spread in such conditions (particularly in cage-free systems and those allowing contact with soil (Lay Jr. *et al.*, 2011)), but only a subset of hens appeared vulnerable to negative consequences for external phenotype and AHN. Compared to good-condition hens, lower *IL-6* mRNA expression in the spleen of birds in poor condition suggests that the latter group were characterized by less efficient innate immune responses to pathogen challenge (Wigley *et al.*, 2002; Swaggerty *et al.*, 2004; Wigley *et al.*, 2006). While these differences may be genetic in origin (Wigley *et al.*, 2002), they may also arise from individual differences in exposure to infectious agents, cumulative experience of other (e.g. social) forms of stress, or an interaction between the two factors.

Both psychological stressors and infections activate the HPA-axis and lead to a release of CORT (Zimomra *et al.*, 2011; Herman *et al.*, 2016). Under normal conditions, low to mid-range

levels of endogenous CORT have potent anti-inflammatory effects, but inappropriate stress responses arising from excessive HPA-axis activation lead to an inflammatory response (Tapp *et al.*, 2019). As with stress, the HPA response to infection is therefore adaptive in the short-term, but deleterious when prolonged (Dhabhar, 2009; Tapp *et al.*, 2019). CORT may exert direct effects on AHN, as a portion (~25%) of hippocampal progenitor cells express GR (Garcia *et al.*, 2004; Lucassen *et al.*, 2010). However, most suppressive effects of CORT on proliferation and survival are thought to be indirectly mediated by the surrounding mature neurons and glia (Song *et al.*, 2002; Wong and Herbert, 2005). In particular, microglia (the primary innate immune cell of the brain) highly express GR and are susceptible to stress-induced priming to future challenge in the presence of elevated CORT levels. As such, successive immune challenges, including illness, stress, and aging, result in microglia exhibiting a hyper-reactive, pro-inflammatory phenotype (Tapp *et al.*, 2019).

When exogenous CORT was administered 24 hours before challenge with *E.coli* lipopolysaccharide (LPS), hippocampal microglia demonstrated a significantly greater expression of inflammatory cytokines compared to that in vehicle-administered controls (Frank *et al.*, 2010). Similarly, when conducted 24 hours after inescapable tail-shock stress, LPS injection significantly increased hippocampal levels of CORT and the inflammatory cytokine IL-1β, relative to vehicle controls (Frank *et al.*, 2012). Moreover, pre-treatment with GR antagonist mifepristone prior to the tail-shocks prevented this exaggerated response to LPS injection. These findings indicate that stress acts directly through microglial GRs to induce a primed inflammatory response (Tapp *et al.*, 2019). Indeed, a systematic review of experimental rodent models concluded that psychosocial stressors (chronic restraint, social isolation and repeated social defeat) significantly and consistently increased microglial reactivity in the hippocampus (and the prefrontal cortex) (Calcia *et al.*, 2016). Pharmacological inhibition of microglia is also protective against the development of stress-induced deficits in spatial memory and depressive- and anxiety-like behaviours, including reduced sucrose preference, social interaction and open field exploration (reviewed in Weber *et al.*, 2016). Other immune cells, such as monocytes (Weber *et al.*, 2016) and astrocytes (Lucassen *et al.*, 2010), are additionally implicated in neuro-inflammatory responses to stress.

Adrenal secretion of CORT has previously been causally implicated in the mechanism by which AHN in mice is reduced in the context of prolonged negative experiences (Lehmann *et al.*, 2013). Studies demonstrating the negative consequences of immune priming by stress for neuronal survival point to likely modulatory mechanisms. Firstly, pre-treatment with chronic stress levels of CORT exacerbated the expression of cytokines IL1β and Tnfα in hippocampal

cultures and contributed to increased excitotoxic neuronal cell death induced by kainic acid (MacPherson *et al.*, 2005). Secondly, LPS-challenge-induced inflammation activated microglia and strongly impaired levels of AHN in rats, while cell numbers were restored by the pharmacological inhibition of microglial activation (Ekdahl *et al.*, 2003). LPS-induced activation of microglia also caused a 35% decrease in AHN ($BrdU^+/DCX^+$ cells) in the mouse DG, which was restored by inflammatory blockade with indomethacin, a common nonsteroidal anti-inflammatory drug (Monje *et al.*, 2003).

Compared to flock-mates in good physical condition, the observed alterations in both expression of mRNA for inflammatory cytokine *IL-6* and composition of the caecal microbiome in poor condition hens lend support to an explanation whereby HPA-axis-mediated inflammation (driven by illness, psychological stress, or a combination of factors) caused a reduction in AHN. This inflammation, and the specific activation of local immune cells such as microglia, may therefore link poor body condition to impaired neural plasticity. Moreover, the research reviewed suggests that, when experienced in combination or succession, stress and physical illness are likely to interact to further exacerbate the dysregulation of both stress responses and immune function (Frank *et al.*, 2012; Tapp *et al.*, 2019). Interestingly, prolonged action of the innate immune system has been observed to induce a program of "sickness behaviour" in animals, which closely resembles the behavioural symptoms of clinical depression exhibited by human patients (Berghman, 2016). These associations provide further support for the relationship between suppressed AHN and negative mood. The same processes may also be implicated in the observed negative relationship between KBF severity and levels of AHN (Chapter 4), wherein the causal factor is more clear.

Injury to a bone leads to an inflammatory response, as immune cells (such as macrophages) are recruited to the site of injury, and multiple factors, including inflammatory cytokines, are secreted (Baht *et al.*, 2018). This intense inflammatory event is required to ensure normal fracture healing, through angiogenesis of blood vessels, repair of the injured tissue, and eventual remodeling. Though the inflammatory response itself is short-lived, the effects of the immune cells extend beyond the early stages of fracture healing (Baht *et al.*, 2018). For example, macrophages and T and B white blood cells play a role during mineralization, as the cartilaginous callus is replaced by the bony callus (Baht *et al.*, 2018). To our knowledge, the study presented in Chapter 4 is the first to explore the relationship between bone damage, chronic stress and AHN. While the inflammatory response to bone injury is generally an adaptive process necessary for healing (Baht *et al.*, 2018), laying hens in non-cage systems typically acquire multiple fractures throughout the laying period, while existing fractures are

repeatedly aggravated during normal hen behaviour, such as perching (Riber *et al.,* 2018). Indeed, a negative consequence of severe KBFs for mobility was demonstrated in Chapter 4, as well as in Rufener *et al.* (2019), wherein hens with worse fractures spent more time on the top tier. In the focal hens from Chapter 4, KBF severity scores tended to become incrementally higher at each successive time-point. This scenario is likely to produce a prolonged or repeated state of inflammation, analogous to that produced by multiple immune challenges (Tapp *et al.*, 2019), and likely to have similar consequences for local immune cells in the brain.

Rodent models wherein chronic neuropathic pain suppressed levels of AHN have generally injured the sciatic nerve (Terada *et al.*, 2008; Mutso *et al.*, 2012; Dimitrov *et al.*, 2014; Romero-Grimaldi *et al.*, 2015), and chronic constriction injury of the sciatic nerve has been shown to induce activation of glial cells and increased levels of mRNA for proinflammatory cytokines (Hernangómez *et al.*, 2016). Generally, physiological and immunological changes occurring after nerve injury converge with those occurring after tissue injury over time, and both trajectories involve the activation of glial cells (Xu and Yaksh, 2011). Inflammation and activation of local immune cells may therefore mediate the negative association between chronic painful experience and AHN. As such, investigation into the possible benefits of inflammatory blockade for laying hen welfare (particularly in non-cage systems) may be warranted.

KBFs may also interact with other sources of stress and ill health to worsen each outcome. Due to dysregulated inflammatory signaling and processing, inflammation caused by bone injury is prolonged and associated with poor healing in conditions of chronic inflammation (such as diabetes, obesity and aging) (Claes *et al.*, 2012; Baht *et al.*, 2018). This suggests that consequences may be worse for individual hens experiencing other sources of stress, or for whole flocks towards the end of lay. In human subjects, dysfunction of the HPA-axis also predicts the likelihood that an (at-risk) individual will develop chronic widespread pain (McBeth *et al.*, 2007). Rodent studies have further highlighted the influence of chronic pain on the consequences of other (positive and negative) experiences for AHN. Neuropathic pain both exacerbates the suppression of AHN caused by chronic immobilisation stress (Romero-Grimaldi *et al.*, 2015) and precludes the stimulatory effects of housing in an enriched environment (Terada *et al.*, 2008). This suggests that KBFs may exacerbate stress from other sources and attenuate the benefits of positive experiences, while stress may worsen the inflammation arising from keel injuries. It has been previously recognised that instances of undesired innate immune activity, such as chronic inflammation, have negative ethical and

economic implications for the farming industry (Berghman, 2016), and the present findings provide further support for an association with poor welfare.

6.3.2 Comparison of effect sizes

For studies in which cells expressing the DCX protein were stained through IHC and quantified in serial HF sections, the effect size of each independent variable was calculated for comparison. This included a previous study conducted in our research group, which exposed adult laying hens to an eight-week program of UCMS and measured AHN in the same manner (Gualtieri *et al.*, 2019). Estimates of the magnitude of influence that the various differential long-term experiences explored had on neuronal differentiation in the laying hen HF are displayed in Table 6.1.

Table 6.1. Partial Eta squared values for estimated effect size of various factors on DCX^+ stained cell densities in the laying hen HF. Values were calculated in repeated measures ANOVAs, accounting for all variables included in the previously reported models. As the effect of UCMS on multipolar cells was specific to the caudal HF, effect sizes in sections from this region were estimated using the "Means" function in SPSS (v.24).

Factor	Study Reference	Multipolar Cell Densities	Bipolar Cell Densities
Final KBF severity score (minimal vs. severe)	Chapter 4	0.514	0.314
Physical body condition (good vs. poor)	Chapter 5	0.144	0.109
Commercial Housing System (multi-tier aviary vs. enriched cage)	Chapter 5	0.005	< 0.001
Genotype/Strain (Hy-Line vs. H&N)	Chapter 5	0.023	0.037
UCMS vs. control housing (caudal HF only)	(Gualtieri *et al.*, 2019)	0.366	0.077

The partial Eta squared values in Table 6.1 indicate that severe KBFs had the strongest effect on DCX^+ multipolar cell densities, accounting for around 51% of the variance between groups. An experimental program of eight weeks UCMS had the second strongest effect, and explained roughly 37% of the variance in multipolar cell densities, though this effect was specific to the caudal HF (the partial Eta squared for the rostral HF was 0.010). Of the factors with statistically significant effects, physical body condition had the weakest effect on DCX^+ multipolar cell densities, accounting for around 14% of the variance. However, conditions were also the least controlled in the corresponding study (Chapter 5), given that hens were sampled directly from commercial housing towards the end of lay. Other unidentified sources of variance in the

lifetime experience of the sampled hens may therefore have contributed to this smaller observed effect size. Nevertheless, when compared to the (non-significant) effect size of housing system (~ 0.5%), the association between body condition and AHN does not appear negligible.

The effect sizes suggest that chronic pain arising from severe KBFs may constitute a source of greater stress for hens than exposure to variable stressors during UCMS, wherein some were physical (e.g. changes in temperature, water spray, food deprivation) and some were more psychological (both social isolation and mixing with unfamiliar birds). The negative influence of chronic pain may therefore exceed that of traditional protocols for inducing depressive-like behaviour in non-human animals. The relative salience and negative valence of the different UCMS stressors from the perspective of the hens, and their consequent individual contributions to overall stressful experience, is unknown, but some may be worse than others. On the other hand, being in poor condition appears to be associated with a level of chronic stress that is milder than an experimental paradigm of UCMS, but notably larger than the difference between living in a multi-tier and an enriched-cage housing system. Many previous studies finding consequences of housing system for welfare made comparisons between conventional battery cages and alternative systems. Generally, conventional cages were found to be associated with relatively negative indicators of welfare, according to outcome measures including H:L ratios (Shini, 2003), tonic immobility (Jones and Faure, 1981; Hansen, 1994; Scott *et al.*, 1998) and fearfulness (Hughes and Black, 1974). It may therefore be that the restricted space and lack of enrichment associated with conventional cages was a source of stress for hens, which exceeded negative experience in other systems. In comparison, the size and resources of modern furnished alternatives (such as the sampled enriched cages, wherein stocking density was similar to the multi-tier aviary) may be adequate to result in a net balance of experiences comparable to certain non-cage alternatives. This is consistent with the previous conclusion that, despite differences in the behaviour of hens in pen and cage environments, physiological and production parameters did not offer any evidence that cage-housing per se was a source of stress, though this was true of high-density caging (Mench *et al.*, 1986).

In combination, these studies suggest that quantitative densities of immature differentiating neurons provide a signal regarding the severity of chronic stressful experience, which permits comparison between conditions. However, research in rodents indicates that while a single experience of stress can cause a transient suppression in AHN (Gould *et al.*, 1997; Tanapat *et al.*, 2001), instances of repeated stress that are mild and/or predictable may not have a negative impact, and may instead stimulate AHN in line with mechanisms of stress inoculation (Parihar *et al.*, 2011; Brockhurst *et al.*, 2015). Such studies suggest that there is a minimum threshold

for the degree of chronic stress necessary to generate a measurable decrease in AHN; at least if this is to be detectable with practical sample sizes. Factors such as housing system may therefore be associated with differential stress of a smaller magnitude, which might still contribute to meaningful differences in welfare, but falls below the level quantifiable through changes in AHN.

In all instances, observed effect sizes were greater for DCX^+ cells with a more mature, multipolar morphology than with a bipolar (or fusiform) morphology. Quantifying cells of the former type may therefore provide the best quantitative proxy of stressful experience and cumulative welfare. The differential sensitivity of these DCX^+ cell types is discussed further in section 6.5.4, in relation to findings in rats and mice. Interestingly, the effects of KBFs and body condition on AHN were observed across the whole HF, while UCMS produced a reduction in DCX^+ multipolar cells that was specific to the caudal subregion. It does therefore not appear that the strength of a chronic stressor determines the spatial extent of its influence on AHN (e.g. through the proportion of glial cells activated), as physical body condition had a smaller effect size than UCMS but was associated with multipolar cell densities in both subregions. Comparative magnitudes of the factors which affected AHN across the whole HF in sections from the rostral and caudal HF are displayed in Table 6.2.

Table 6.2. Partial Eta squared values for the estimated effect sizes of factors which significantly influenced DCX^+ stained cell densities over the whole HF, calculated separately for sections from the rostral and caudal subregions. Effect sizes were estimated using the "Means" function in SPSS (v.24), with comparisons made between groups defined by the specified factor.

	Multipolar Cell Densities		Bipolar Cell Densities	
Factor	Rostral HF	Caudal HF	Rostral HF	Caudal HF
Final KBF severity score (minimal vs. severe)	0.182	0.776	0.196	0.272
Physical body condition (good vs. poor)	0.240	0.065	0.095	0.040

Like the specific influence of UCMS in the caudal HF (Gualtieri *et al.*, 2019), the considerably greater effect of KBF severity group on DCX^+ cell densities in this subregion (Table 6.2) lends support to the conclusion that the caudal chicken HF is preferentially sensitive to chronic stress. However, the magnitude of the effect of physical body condition was instead greater in the rostral HF. Previously, feed restricted broiler chickens were also found to have lower numbers of one-week old proliferating neuronal cells than *ad libitum* fed birds in the rostral HF alone, in an effect attributed to the stress of chronic hunger (Robertson *et al.*, 2017). Collectively, these findings suggest that whether AHN is impaired across the extent of the avian HF, or only within a particular subregion, by chronic stress, and which of the subregions is preferentially

involved in the latter instance, depends upon the exact modality of the associated stressor(s). It is possible that psychological stressors have a particular suppressive effect on temporal AHN, while physical stressors (e.g. poor health/recurrent infection) also affect septal AHN and thus influence proliferation and survival over the whole HF. The UCMS protocol included psychosocial stressors in the form of social mixing and social isolation, while the unpredictable schedule may have led to anticipatory stress. It may be that these components had the strongest negative influence on levels of AHN, and the fact that body mass was not influenced by the UCMS program may point to less of a global effect on overall (physical + mental) state. Though changes in inflammatory gene expression and caecal microbiome composition may also be attributable to psychological stress (e.g. arising from subordinate social status), the use of phenotypic criterion for selection likely led to a greater contribution of physical factors (relating to health) in the housing systems study.

It is also possible that AHN in a particular region is affected first, but with time, the process is downregulated over more of the structure. Such a time-dependent response was demonstrated for the effect of antidepressant treatment on AHN in rats, wherein a three week course of agomelatine stimulated the proliferation and survival of new-born cells in the temporal hippocampus alone, but six weeks of treatment had this effect in both subregions (Banasr *et al*., 2006). This explanation would be consistent with the shorter timescale of the eight-week UCMS (Gualtieri *et al.*, 2019) and five-week food restriction (Robertson *et al.*, 2017) manipulations, whereas the commercial hens had lived in their adult housing conditions for 49/50 weeks, and most hens had KBFs for ~34 weeks. The hens in the KBF and housing system studies were also 61 to 66 weeks old when killed, compared to only 26 weeks old for the UCMS study (and 12 weeks for the feed-restricted broiler chickens). Aging is associated with increasing inflammation and HPA-axis dysregulation (Sapolsky *et al.*, 1986; Tapp *et al.*, 2019) which may contribute to an exacerbated negative impact of experienced stress on AHN, perhaps leading to vulnerability across the entirety of the HF. Future studies quantifying AHN at different time points, and in different age hens, may seek to address these possibilities. The alternative explanation is that our rostral/caudal division is not (at least entirely) equivalent to the septal/temporal dissociation in the mammalian hippocampus. Evidence for the differential sensitivity of these HF subregions is further considered in section 6.5.2, in relation to homologies with rats and mice.

Collectively, these findings strongly support the implementation of interventions to reduce the occurrence of KBF in order to improve the cumulative experience of commercial laying hens. Measures to limit circumstances which lead to poor body condition in commercial flocks, such

as infection and feather-pecking, may also have benefits, though these may be less dramatic in isolation. The results of the previous UCMS study (Gualtieri *et al.*, 2019) might also suggest that conducting husbandry procedures according to a predictable schedule could have positive implications for laying hen welfare. In circumstances where multiple different (physical) measures of laying hen welfare are collated to produce an aggregate score, the present findings may also provide information regarding how some of these should be weighted (discussed in Nicol *et al.*, 2009).

6.3.3 Positive experiences

PCNA mRNA expression in the rostral subregion of the HF, hypothesized to be homologous to the septal rodent hippocampus (Smulders, 2017), correlated positively with the proportion of time that hens spent in the uncovered outdoor areas of a free-range system. It therefore appears that proliferation in the laying hen HF is upregulated by environmental complexity, equating to size of the utilized home range and the associated spatial-cognitive and navigational demands. The experience of storing and retrieving food caches is similarly known to stimulate proliferation in Marsh tits (Patel *et al.*, 1997). Though the primary effect is to enhance the survival of immature neurons (Van Praag *et al.*, 1999), environmental enrichment (EE) programs in rodents have also been associated with increased numbers of proliferative progenitor cells (Bruel-Jungerman *et al.*, 2005; Steiner *et al.*, 2008). Arguably, hens that made use of the additional space afforded by the external areas beyond the barn and adjacent winter-garden, and experienced these more unpredictable environments, were privy to a greater level of enrichment. In rats and mice, EE has anxiolytic and antidepressant effects (Benaroya-Milshtein *et al.*, 2004; Fox *et al.*, 2006; Llorens-Martín *et al.*, 2007; Hendershott *et al.*, 2016), indicative of an association with a positive affective state. However, in Chapter 3, durations of tonic immobility (TI), which are believed to reflect fearfulness or anxiety in chickens (Gallup, 1979), were not associated with ranging, but appeared to account for independent variation in individual HF proliferation that was likely related to coping style. Melleu *et al.* (2016) conducted an enriched housing manipulation for adult pigeons that resembled rodent studies (e.g. providing larger cages containing salient objects and affording visual contact with neighbours), and observed greater subsequent numbers of DCX^+ neurons compared to control birds. However, the enriched group also displayed prolonged TI durations, which did not correlate with cell counts on an individual basis. Therefore, while studies in avian species demonstrate that AHN levels are responsive to cognitive stimulation, it is not clear whether or not these scenarios are associated with a corresponding positive subjective experience for the birds.

In Chapter 2, experience of the "Preferred" experimental housing condition, which was designed with the intention of best satisfying the preferences of the majority of birds, was reflected neither by gene expression related to AHN or glucocorticoid regulation, nor by existing physiological measures of welfare (serum CORT, H:L ratio, etc.) or a behavioural measure of anhedonia (mealworm consumption). While some of the differences between conditions were arguably subtle variations in pen furniture, the "Preferred" conditions were accompanied by the provision of discrete positive reinforcers, mainly in the form of portions of treat foods. Pharmacological manipulation of anticipatory behaviour in chickens has demonstrated that such high value foods (e.g. mealworms) activate neural circuits involved in the subjective hedonistic experience of reward (Taylor *et al*., 2020). Therefore, hens in the "Preferred" housing are known to have had (albeit brief) positive affective experiences several times a day. Unfortunately, none of the outcome measures taken reflected differing consequences of the sum of experiences in each housing condition. As discussed in section 6.2, the null findings may relate to limitations of transcription of the *DCX* gene as a proxy for neuronal differentiation. However, it is also possible that AHN in chickens, and possibly birds in general, is a better biomarker of chronic stress than of positive experiences. Future studies must definitively elucidate whether AHN levels in poultry are sensitive to long-term experiences of known positive valence. Generally, it is difficult to ascertain through tests of behavioural needs and priorities (as reviewed in Weeks and Nicol, 2006) which experiences chickens find intrinsically rewarding/pleasurable, as opposed to those which merely constitute deficit needs and cause negative affect if absent or not possible. Further well-designed manipulations of reward circuits (as in Taylor *et al*., 2020) may be necessary to determine which of these categories various experiences, such as the use of dust baths or perches, belong to. Determining whether the association between numbers of immature neurons and affective experience in chickens is truly bi-directional might also be aided by a 2x2 design employing experimentally controlled chronic stressors (such as the UCMS protocol in Gualtieri *et al.*, 2019) and treatment with an antidepressant agent, followed by morphological quantification of AHN. Even so, the efficacy of antidepressant agents in chickens is currently unknown, and interpretation of these results would be dependent upon concurrent behavioural measures to confirm associations with mood.

6.3.4 Individual differences in personality & cognition

In Chapter 3, expression of *PCNA* mRNA over the whole HF was found to co-vary positively with durations of tonic immobility, consistent with the previously reported association between hippocampal proliferation and individual differences in reactive/proactive behavioural strategy

(or coping style) in rodents and fish (Lemaire *et al.*, 2008; Vindas *et al.*, 2017). In Chapter 2, choice of housing was associated with serum CORT, *MR*/*GR* and *PCNA* mRNA expression. Hens from both housing conditions ("Preferred" & "Non-Preferred") that made the minority choice had higher levels of serum CORT, while hens from "Non-Preferred" housing that made the minority choice had lower *MR*/*GR* ratios in the rostral HF. Expression of *PCNA* mRNA was higher in hens from both housing conditions that chose in favour of the baseline conditions. In line with findings from Chapter 3, this latter result may relate to the association between hippocampal proliferation and behavioural strategy. Proactive individuals tend to behave in a rigid, routine-like manner and are less attentive to changes in the environment (Benus *et al.*, 1991; Coppens *et al.*, 2010; Höglund *et al.*, 2017). When presented with the choice, these hens may have therefore opted to resume their habitual behaviour in the housing environment that they had lived in for the previous 26 weeks. In contrast, higher levels of AHN in reactive individuals has been linked to a greater degree of behavioural flexibility (McGraw, 1973; Benus *et al.*, 1991; Zidar *et al.*, 2019). Reactive individuals are more likely to attend to changes in the environment, take time to gather information, and change their behaviour (Coppens *et al.*, 2010; Höglund *et al.*, 2017). These birds may therefore have chosen in favour of the more novel/less familiar baseline housing environment. It is also possible that the reactive individuals preferred the specific properties of the baseline environment. However, in the case of hens from the "Preferred" housing, the difference in conditions was perhaps unlikely to have been salient enough to induce an automatic, unlearnt preference. For example, the "Preferred" and baseline pens were both lined with wood shavings, but these were deeper in the former. As proliferation may respond fairly quickly to stress (e.g. Malberg and Duman, 2003), it is also possible that individual differences in *PCNA* expression could have been determined by variable stress responses to capture. However, as reactive individuals exhibit greater responses to acute stress (Koolhaus *et al.*, 1987; Cockrem, 2007), these hens would have been predicted to experience a greater suppression of proliferation and consequently exhibit lower, rather than higher, *PCNA* expression, were this experience highly influential.

Instead of relating to familiarity/temporal experience of the housing options, or the particular environment chosen, concentrations of CORT in the blood serum related to whether hens made the majority or minority choice. In birds from both housing systems, the minority choice was the choice in favour of the objectively worse of the options provided. Though individual hens differ in their preferences for particular types of inanimate housing environment, for reasons which are unclear (Nicol *et al.*, 2009), the minority hens from the "Preferred" and "Non-Preferred" housing made choices in favour of different conditions, and so are unlikely to

represent a single group with common traits. The preferences exhibited could be interpreted as discontentment with the experimental housing for the P hens that chose the baseline conditions, and contentment with the experimental housing for the NP hens that chose the NP conditions. However, a reduced *MR*/*GR* ratio in the latter group suggests that these individuals may have actually experienced the most stress (Dickens *et al.*, 2009), meaning their choice could actually represent a learned helplessness-type response.

In Chapter 5, individual differences within two commercial housing systems also had a greater influence on AHN than between-group differences associated with each environment. Though the responsivity of DCX^+ cell numbers in the laying hen HF to experimental stress has been demonstrated (Gualtieri *et al.*, 2019), and the direction of causation appears clear for the KBF study (Chapter 4), a contribution of intrinsic individual differences in traits associated with AHN, such as reactivity, to cumulative stress and eventual physical condition cannot be ruled out. However, in this case, reactive individuals with higher AHN and cognitive flexibility would be expected to exhibit stronger responses to stress, which might chronically lead to a higher level of HPA-axis dysregulation and inflammation. As such, the predicted interaction between basal AHN and stress reactivity would be compensatory, rather than exacerbatory, and is unlikely to explain the results observed.

Together, the studies in Chapters 2, 3 and 5 highlight the importance of considering the implications of individual differences within groups of birds for laying hen welfare. This individual variation may relate to intrinsic traits, or to differential experience, and the individual by environment interaction is doubtless influential in terms of overall welfare. The findings therefore lend additional support to efforts to selectively breed hens for robustness and resistance to psychological and immune stress (e.g. Gross and Siegel, 1985; Rauw and Gomez-Raya, 2015), by highlighting consequences for the cumulative affective experience of the individual birds. For example, group selection for reduced levels of feather-pecking and negative social interactions caused positive changes in behavior, stress physiology and immunology, increased resistance to heat stress, and reduced mortality to levels comparable to individually housed birds over five generations (Muir *et al.*, 2014). The *PCNA* results in Chapter 3 also indicate that collecting other measures from focal hens may allow some degree of differentiation between the contribution of personality traits and experience to hippocampal plasticity.

6.4 Relation of AHN to Existing Measures of Laying Hen Welfare

In the avian brain, neuronal progenitors begin to migrate away from the lateral ventricle wall after three days (Alvarez-Buylla *et al.*, 1990), while DCX-expression persists for 25 to 30 days (Balthazart *et al.*, 2008). The most mature DCX^+ cells exhibit a multipolar morphology and are presumed to be settling into the pre-existing circuity (Boseret *et al.*, 2007; Balthazart *et al.*, 2008). DCX IHC thus labels a mixed population of immature neurons, probably ranging from three to 30 days old. As such, the method integrates levels of proliferation, and the proportional survival of immature neurons through on-going differentiation, over the previous month. However, studies in rodents indicate that the modulatory effect of valenced experiences on future proliferation, neuronal differentiation and survival may persist for a longer period, even when the experience is discontinued in the interim. For example, numbers of $BrdU^+$ proliferative cells did not differ when measured immediately after 21 days of restraint stress, or when this negative experience was succeeded by 10 days of intervention-free standard housing (Veena *et al.*, 2008), indicating that no spontaneous recovery had occurred by this time. Similarly, DCX^+ cell numbers were still reduced at the end of a 3-month individual housing period that followed a short (5-day) period of resident-intruder defeat (Van Bokhoven *et al.*, 2011), though it is possible that this isolated housing also constituted a stressor. Numbers of both $PCNA^+$ proliferative cells and DCX^+ immature neurons also remained suppressed for at least three weeks after the reversal of a neuropathic injury (Dimitrov *et al.*, 2014). Finally, following six to eight weeks of UCMS, the survival of $BrdU^+/NeuN^+$ cells over the next, intervention-free, month was also decreased (Surget *et al.*, 2011), though survival two months after the cessation of stress was no longer affected (Elizalde *et al.*, 2010). However, rapid recovery of AHN has been observed following other paradigms, for example with proliferation normalised 24 hours after 10 days of social defeat (Lagace *et al.*, 2010). The duration of influence on levels of AHN may therefore depend upon specific characteristics of the experience in question. While longitudinal data on the severity of KBFs was collected, it is not known for how long fractures continue to be a source of pain to hens, while damage scores tended to worsen incrementally. However, changes in KBF severity as early as ~37 weeks previously predicted AHN at the end of life. In Chapter 5, hens sampled from the commercial housing where they had spent the previous 49 or 50 weeks remained on-farm until the morning of tissue collection. This study does therefore not offer any insights into the broader time-window over which AHN in chickens might integrate cumulative experience. While the findings of Chapter 4 suggest that this may exceed the maturation period of the quantified cell population, hens continued to have KBFs throughout their lifetimes, and it was not possible to

assess whether the suppressive effect on AHN would have persisted in the absence of this stressor. Future experimental work in chickens might address this question, by leaving intervention-free delays between controlled stressful experiences and HF tissue collection.

Assuming that the timespan for which post-mortem DCX^+ cell densities reflect integrated retrospective experience (i.e. the latency until the effects of past stresses or positive experiences are no longer detectable) does not exceed one month, this biomarker is still likely to be more informative in terms of whether a group of animals had a "life worth living" than measures of acute stress. While concentrations of CORT remain the most commonly used measure of poultry welfare, concentrations in plasma peak around 30-50 minutes after commencement of stress (Droste *et al.*, 2008) and drawing blood from broiler chickens more than two minutes (150 or 180 seconds) after capture produces samples with higher CORT concentrations (Chloupek *et al.*, 2011). Therefore, though basal levels of CORT may be altered by chronic stress-induced dysregulation of the HPA-axis (Tapp *et al.*, 2019), studies measuring CORT in laying hens are susceptible to strong influence by the methods of capture or handling employed (as in Mench, 1986). Additionally, dysregulation of basal CORT secretion through the HPA-axis may occur in either direction. For example, post-traumatic stress disorder in human patients, and in mouse models, is characterized by inadequate release of CORT in response to stressors (Tapp *et al.*, 2019), while suppressed basal levels of CORT were observed in hens exposed to UCMS (Gualtieri *et al.*, 2019), presumably as a result of repeated stimulation of the HPA-axis (as in Rich and Romero, 2005; Dickens *et al.*, 2009). Like excessive CORT, insufficient CORT is also inflammatory, as the immunosuppressive effects of the normal levels secreted to restore homeostasis are absent (Dhabhar, 2009; Tapp *et al.*, 2019). Therefore, while levels of CORT and other indicators of HPA-axis activity may not quantitatively or directionally reflect stressful experience, an apparently reliable suppressive effect of dysregulation of these mechanisms on AHN (e.g. through activation of local immune cells, among other mechanisms) may confer advantages as a biomarker of welfare.

The baseline comparison in pullets of two genotypes conducted in Chapter 5 also suggests that numerical comparison of cell counts between strains of brown hen may be possible. Comparatively, certain strains of mice have been found to differ in their baseline levels of AHN (Kempermann *et al.*, 1998; Kempermann *et al.*, 2006). It may be interesting to compare baseline AHN between laying hen pullets of white and brown varieties, as studies have reported differences in several measures of stress and fearfulness, including fear of humans (Murphy, 1976), docile behaviour (Murphy, 1977) and TI and plasma CORT responses to acute handling stress (Fraisse and Cockrem, 2006). As white strains of hen appear to be generally more fearful

and susceptible to stress, such studies raise the question of whether these traits are accompanied by lower levels of basal AHN, which confers resilience (Levone *et al.*, 2015).

AHN may complement existing measures of longer-term or chronic stress in poultry, such as H:L ratios and measures of fluctuating asymmetry. The comparative reliability and sensitivity of these novel and existing methods remains to be fully determined. Previously, however, AHN has reflected differential stressful experience of chickens (subject to UCMS) where durations of TI and H:L ratios did not (Gualtieri *et al.*, 2019). In the present work, durations of TI were also not related to the proportional time spent by hens in outdoor areas, whereas proliferative gene expression in the rostral HF was. A former study found that 72 hours of fasting or frustration of feeding resulted in elevated H:L ratios, but had no effect on TI responses. Instead, individual differences in pre-treatment TI durations positively predicted the magnitude of hens' H:L responses to stress (Jones, 1989). Consistent with high levels of heritability (Gallup, 1974), TI may generally constitute a better measure of individual differences in susceptibility to stress (reactivity), than of stressful experience relating to environmental conditions. It is possible that AHN may provide additional quantitative sensitivity beyond existing measures of chronic stress, for example by reflecting individual differences in the severity and duration of KBFs. In comparison, while fluctuating asymmetry was found to be lowest in the population of hens with the highest aggregated welfare score, there was no association at the level of the individual (Tuyttens *et al.*, 2008). Overall conclusions drawn from the comparison of furnished cage and non-cage (multi-tier aviary) commercial housing through quantification of AHN in Chapter 5 were similar to those arising from an integrated welfare score which collated numerous physical, physiological and behavioural parameters (Rodenburg *et al.*, 2008).

6.5 Functional Similarities between AHN in the Avian and Mammalian Hippocampal Formations

The rationale underlying use of AHN as a biomarker of subjective long-term experience in poultry arose from associations of the process with self-reported human mood, in the context of major depressive disorder and antidepressant treatment (Miller and Hen, 2015). This relationship is mirrored strongly by the co-variance of AHN and anxiety- and depressive-like behaviour in rats and mice with chronic stress (in a negative direction) and antidepressant treatment, EE and exercise (in a positive direction). Previous studies in songbirds have demonstrated that AHN in avian species is similarly upregulated by use of spatial-cognitive processing and memory capacities (Patel *et al.*, 1997; LaDage *et al.*, 2010), and downregulated by chronic stress (Barnea and Nottebohm, 1994; LaDage *et al.*, 2010; Roth *et al.*, 2012).

Additionally, two studies have indicated that AHN in commercial strains of chicken is suppressed by experiences associated with chronic stress (Robertson *et al.*, 2017; Gualtieri *et al.*, 2019). Overall, the present research has provided additional support for the hypothesis that adult neurogenesis in the laying hen HF is modulated by conditions and experiences similar to those that regulate its levels in humans, primates, rats and mice.

6.5.1 Directional modulation of AHN in the laying hen brain according to valence

Firstly, the directional sensitivity of adult neurogenesis in the laying hen HF to negatively-valenced experiences has been further demonstrated, in relation to KBFs and poor physical condition. The downregulation of AHN in the avian brain by chronic pain parallels experimental findings in rats and mice (Mutso *et al.*, 2012; Dimitrov *et al.*, 2014; Romero-Grimaldi *et al.*, 2015; Zheng *et al.*, 2017), and does not appear to have been previously demonstrated for a species of bird. While several factors may contribute to poor body condition in commercially housed hens, the phenotypic criteria utilized are known to be associated with negative experiences and outcomes for the birds. Low body mass is a consequence of administration of exogenous CORT (El-lethey *et al.*, 2001) and ACTH (Thaxton and Puvadolpirod, 2000), immune stress (Yang *et al.*, 2011) and transport stress (Karaman, 2009). Meanwhile, poor plumage condition is associated with nervousness (Ouart and Adams, 1982; Na-Lampang and Craig, 1990), receipt of pecks from conspecifics (Bilcik and Keeling, 1999) and impaired thermoregulation (Herremans and Decuypere, 1986), and a pale comb has been linked to subordinate social status (Bradshaw, 1992; Cloutier *et al.*, 1996). Coupled with the findings of altered caecal microbiota compositions, inflammatory cytokine (*IL-6*) mRNA expression and enlarged spleens (for poor condition birds from the enriched cage system), there is good evidence to support the assumption that poor condition hens displaying relatively lower numbers of DCX^+ neurons were also subject to negative stress. Several studies now therefore support the conclusion that AHN in domestic chickens is attenuated by chronic stressful experience (Robertson *et al.*, 2017; Gualtieri *et al.*, 2019; Chapter 4; Chapter 5). Given the apparent similarity to outcomes of rodent models of stress, the present research supports the conclusion that the regulation of AHN in modern mammals and birds derives from a process that was already established in their common ancestor, over 300 million years ago (Striedter, 2016).

Though findings regarding transcription of the *DCX* gene may not be trustworthy, the positive relationship between *PCNA* expression in the rostral HF and time spent in the outdoor areas of a free range system is consistent with the stimulatory effect of spatial-cognitive challenge on

AHN in the avian brain (Patel *et al.*, 1997; LaDage *et al.*, 2010). In the same study, no relationship was found between the number of transitions that hens made between the four discrete areas (barn, winter-garden, stone yard and range). Though this measure comprised only a very crude proxy for exercise, activity in terms of the number of vertical aviary transitions made by hens within the multi-tier (indoor only) system in Chapter 4 also did not correlate with individual DCX^+ cell densities in the HF. This latter measure still did not account for horizontal activity, and future studies may seek to link AHN with more complex longitudinal assessments of hen movement, which account for consistency and changes in behavioural patterns (as in Rufener *et al.*, 2018). Previous studies have also not found an association between exercise and AHN in birds. Firstly, flight tunnel exercise did not upregulate DCX^+ cell numbers in European Starlings (*Sturnus Vulgaris*) in comparison to conspecifics deprived of this experience (Hall *et al.*, 2014). Secondly, while AHN in mountain chickadees was upregulated by cognitive challenge, it was not influenced by physical activity, as demonstrated in a control group of birds that travelled an equal distance in the testing room but were not given food to cache and retrieve (LaDage *et al.*, 2010).

In contrast, studies have revealed a strong positive linear relationship between the levels of running exercise conducted by individual rats and mice and measures of AHN (Allen *et al.*, 2001; Rhodes *et al.*, 2003). Molecular evidence, angiogenesis and tests of cognitive function suggest that AHN in humans is also enhanced by exercise (reviewed in Lei *et al.*, 2019), indicating that the relationship is probably not unique to rodents. However, it remains possible that the regulatory association between exercise and AHN is a more recent evolutionary addition to the mammalian lineage, occurring since their last common ancestor with birds. The high spontaneous levels of running enacted by rats or mice when presented with a wheel (Garrett *et al.*, 2012) may suggest that they (along with people) enjoy this activity, while birds may not find exercise similarly rewarding. There is some evidence that species-specific preferences for particular modalities of exercise determine their associations with AHN. Unlike running in a wheel or on a treadmill (e.g. van Praag *et al.*, 1999; Garrett *et al.*, 2012; Kim *et al.*, 2017), forced swimming did not increase AHN in mice (van Praag *et al.*, 1999), while completing a water maze resulted in a decrease in AHN, which was prevented by the opportunity for habituation to the pool (Ehninger and Kempermann, 2005). Anaerobic resistance training, which involved climbing a vertical ladder while fitted with weights, also did not enhance proliferation, DCX-expression, or neuronal survival in rats (Nokia *et al.*, 2016). Therefore, in contrast to the positive affective experience associated with running, forms of exercise such as swimming may instead be naturally stressful for rodents, and do not appear to

upregulate AHN. Determining which, if any, forms of physical activity chickens enjoy enacting may be necessary to inform predictions regarding relationships with AHN. For most mammalian species, movement in contact with the ground is the only regular mode of locomotion, and this may not be entirely analogous to either flight or walking behaviour in birds. Birds also do not tend to conduct locomotor behaviour in isolation (e.g. in a fixed position, as with a running wheel), and walking is often concomitant with foraging for chickens (Chielo *et al.*, 2016). Given that the use of spatial memory capacities stimulates AHN, navigation towards a goal location within a known terrain (e.g. to return to the barn from the outdoor range) involves indistinguishable cognition and movement. Operationally quantifying the consequences of exercise for neural plasticity may therefore be more challenging in avian species.

6.5.2 Existence of a septo-temporal gradient across the laying hen HF

Gene expression and connectivity differ across the septo-temporal (or longitudinal) axis of the mammalian hippocampus, while lesion studies indicate specialization of function (Moser and Moser, 1998; Herman and Mueller, 2006; Fanselow and Dong, 2010; Strange *et al.*, 2014). Basal differences in rates of AHN also exist across the septo-temporal axis of the hippocampus in rats and mice. Numbers of DCX^+ immature neurons, reflecting ongoing neuronal differentiation, are higher in the septal dentate gyrus (Snyder *et al.*, 2009; Tanti *et al.*, 2013), but numbers of incorporated adult-born mature neurons are greater in the temporal subregion than in the septal DG (Tanti *et al.*, 2012; Tanti *et al.*, 2013; Anacker and Hen, 2017). Accounting for KBF severity scores revealed an overall greater density of new multipolar neurons in the caudal HF than in the rostral subregion, which was also observed in both the adult hens and laying hen pullets sampled in Chapter 5, and is consistent with previous work in our research group (Gualtieri *et al.*, 2019). This regional gradient has been observed in other avian species, with the same pattern present in zebra finches (Barnea *et al.*, 2006) and two species of North American blackbird (Guigueno *et al.*, 2016). However, levels of proliferation are conversely higher in the rostral VZ of marsh tits (Patel *et al.*, 1997), and rates of neurogenesis are higher in the rostral HF of black-capped chickadees than at the caudal pole (Barnea and Nottebohm, 1994). The gradient in production of new neurons across the longitudinal axis of the avian HF thus appears to differ depending upon the species in question.

Rodent studies that have differentiated between tissue sections across the septo-temporal axis of the HF have typically observed subregional specialization in the sensitivity of AHN to various experiences. In particular, AHN in the temporal hippocampal subregion appears

especially vulnerable to suppression by chronic stress, with reductions in proliferation (Jayatissa *et al.*, 2006) and the survival of immature neurons (Hawley and Leasure, 2012; Lehmann *et al.*, 2013) limited to this region in some paradigms. Studies measuring multiple stages of AHN in the same animals have produced results that are somewhat more mixed. UCMS was found to reduce the 24-hour proliferation of BrdU-labelled cells only in the temporal hippocampus (Tanti *et al.*, 2012), while numbers of $BrdU^+$ cells labelled between 24 and 72 hours previously were reduced in both subregions (Elizalde *et al.*, 2010). Both studies observed a suppression in the four-week survival of $BrdU^+/NeuN^+$ neurons that was restricted to the temporal subregion (Elizalde *et al.*, 2010; Tanti *et al.*, 2012). However, a further exploration found that UCMS produced a uniform decrease in Ki-67^+ cell proliferation, $BrdU^+$ four-week survival and numbers of immature post-mitotic ($DCX^+/Prox1^+/calretinin^+$) neurons across the whole hippocampus, while downregulating the number of $DCX^+/Prox1^+/calretinin^-$ progenitor cells only in the temporal DG (Tanti *et al.*, 2013). The restorative effects of antidepressant treatment were found to reverse decreases in the various stages of AHN in all regions that these were observed, i.e. specifically in the temporal DG (Tanti *et al.*, 2012) and across the entire septo-temporal axis (Tanti *et al.*, 2013).

These findings highlight the fact that AHN in the temporal subregion of the mammalian hippocampus is generally more vulnerable to attenuation by stress, but that whether a differentiated or homogenous pattern is detected across the septo-temporal axis may depend upon the stage of neuronal development that is quantified. Previously, UCMS was found to suppress densities of DCX^+ multipolar cells within the caudal laying hen HF alone (Gualtieri *et al.*, 2019), demonstrating that the methodology similarly applied to the present studies is capable of revealing a subregional gradient in responsivity of adult-born cell numbers. However, both severe KBFs and poor physical body condition were associated with lower numbers of immature differentiating DCX^+ neurons over the whole HF. The associated effect size was greater in the caudal HF in the former instance, but stronger in the rostral HF in the latter (see section 6.3.2). Studies in rodents do not appear to have compared the relative influence of physical and psychological stressors on AHN across the septo-temporal axis, and this aspect of stress modality might influence the localization of effects on neuronal differentiation and survival. Like KBFs, a model of chronic inflammatory pain in mice was found to downregulate proliferation and neuronal differentiation in both the septal and temporal DG (Zheng *et al*., 2017). Interestingly, however, selective ablation of AHN in either subregion produced dissociated impairments in the recovery of symptoms mediated by EE and running exercise. Specifically, AHN in the temporal DG participated in alleviating the perceptual and

anxiolytic components of chronic pain, whereas AHN in the septal DG was involved in the restoration of cognitive function. It therefore appears that severe forms of stress (such as chronic pain) influence several different functional hippocampal processes, whereas the extent of their various involvements in response to milder stressors (e.g. UCMS and poor physical condition) may relate to stimulus modality.

Nevertheless, sensitivity of AHN to individual differences in certain aspects of KBFs was observed to be greater in the caudal HF subregion. Not only were associations between DCX^+ cell counts and individual KBF severity scores stronger in this region, but the time point at which the first KBF was acquired tended to co-vary with caudal cell densities, with no relationship to neuronal differentiation in the rostral subregion. Similarly, though antidepressant treatment upregulated AHN in both hippocampal subregions of rats exposed to chronic stress, $BrdU^+$ cell numbers in the temporal DG alone differentiated between individuals exhibiting recovery from anhedonia and behavioural non-responders, and positively predicted individual differences in sucrose intake (Jayatissa *et al.*, 2006). The results of Chapter 4 therefore somewhat support a gradient of vulnerability to stress across the longitudinal axis of the avian HF which is homologous to mammals, and would be consistent with the greater regulatory control over the HPA-axis that is exerted by electrical stimulation of the caudal pigeon HF (Bouillé and Baylé, 1973).

On the other hand, the relationship between expression of proliferative marker *PCNA* and time spent ranging in areas external to the barn (and adjoining winter-garden) was restricted to the rostral subregion of the HF. Several studies have found that housing mice in an enriched environment preferentially stimulates AHN in the septal DG. EE has been reported to increase proliferation and the proportion of post-mitotic immature neurons (Tanti *et al.*, 2013), densities of DCX^+ cells (Gualtieri *et al.*, 2017), and the four-week survival of new neurons (Nollet *et al.*, 2012) in the septal hippocampus alone. However, one study found that numbers of new neurons surviving six-weeks after EE were uniformly distributed across the septo-temporal DG (Tashiro *et al.*, 2007). Studies in food-hoarding birds also point to preferential involvement of the septal HF in spatial-cognitive processing. For instance, an increase in neuronal density occurs specifically in the rostral third of the black-capped chickadee HF prior to hoarding season (Smulders *et al.*, 1995). Additionally, expression of immediate early gene ZENK occurs in more neurons from the rostral than the caudal HF as these birds fly around a room to forage, hoard and/or retrieve food (Smulders and DeVoogd, 2000). A preferential relationship between the time spent in outdoor areas, requiring navigation of an effectively larger home range, and AHN

in the septal avian HF is thus consistent with current understanding of functional hippocampal specialisation in mammals and birds.

When comparing KBF groups in Chapter 4, hens with minimal fractures alone had more new multipolar neurons in the caudal HF than the rostral region, whereas hens with severe KBFs exhibited no subregional difference. Similarly, while control hens previously had a higher density of DCX^+ multipolar cells in the caudal HF than the rostral subregion, there was no subregional difference for hens exposed to UCMS (Gualtieri *et al.*, 2019). As discussed, these findings indicate preferential sensitivity of neuronal differentiation in the temporal (caudal) chicken HF to stress. Interestingly, however, while adult wild-caught chickadees instead exhibit a higher level of AHN in the rostral HF subregion, this gradient was eliminated in birds kept in captivity for six weeks at the same time of year (Barnea and Nottebohm, 1994). Though further exploration is required, it could be that certain sources of chronic stress, such as KBFs, UCMS and captivity, have some form of homogenising effect on species-specific subregional rates of AHN. In Chapter 5, hens in poor physical condition still had higher DCX^+ cell densities in the caudal HF, which may relate either to the smaller associated effect size, or to dissociated regulatory mechanisms linking stress and body condition.

It may be necessary to confirm the specificity of some of the observed effects to the HF, by quantifying AHN in a control region of the telencephalon. However, where effects were limited to, or stronger within, a single HF subregion, this supports a relationship between experience of the environment and adult neurogenesis which is specific not only at the structural level, but at the sub-structural level, rendering such a control less necessary.

6.5.3 Differences between the hemispheres

Evidence from mouse models of neuropathic pain suggests that modulation of AHN in the mammalian brain is not lateralized, as damage restricted to a single paw produces a comparable bilateral suppression in numbers of immature neurons (Mutso *et al.*, 2012). In pigeons, studies highlight notable functional lateralization within the avian forebrain (Bingman *et al.*, 2006; Jonckers *et al.*, 2015), which may differ from that in mammals given that birds lack a corpus callosum (Rogers, 1986). Previously however, quantification of AHN in passerine birds has also revealed no hemispheric differences in numbers of surviving new neurons. This is true for the number of surviving (thymidine- or BrdU-labelled) neurons in the HF of both wild and captive black-capped chickadees (Barnea and Nottebohm, 1994; Tarr *et al.*, 2009), and for the number of DCX^+ immature neurons in the mountain chickadee HF (LaDage *et al.*, 2010). In the present IHC studies, DCX^+ cells were quantified in the HF from a single hemisphere for

each focal bird, in a manner that was balanced between the left and right hemisphere within groups of hens. Because quantification of cells in a single hemisphere per bird meant that it was not possible to distinguish potential lateralization from individual differences, an effect of hemisphere was not included in the reported statistical models. However, when this factor is explored alongside the existing variables, DCX^+ cell densities do not differ between HF sections from the left and right hemisphere, either for the adult laying hens or the 14-week-old pullets (effect of hemisphere - Chapter 4: multipolar $F_{1,17.0} = 0.18$, $p = 0.675$, bipolar $F_{1,17.1} = 3.05$, $p = 0.099$; Chapter 5, adult hens: multipolar $F_{1,38.6} = 0.26$, $p = 0.776$, bipolar $F_{1,38.7} = 0.54$, $p = 0.585$; pullets: multipolar $F_{1,19.2} = 1.09$, $p = 0.310$, bipolar $F_{1,20.3} = 1.02$, $p = 0.324$). While a within-bird comparison of DCX^+ densities in the two hemispheres would be required to draw a definite conclusion, the lack of a hemispheric difference in AHN is consistent with findings in chickadees, and with the general lack of structural asymmetry reported within the avian brain, despite much functional asymmetry (Rogers, 1986).

In Chapter 2, the association of *PCNA* mRNA expression with choice (for experimental versus baseline conditions) was specific to the left hemisphere. Previous literature suggests that reactive and proactive coping behaviours may be mediated by lateralised networks, as proactive coping behaviours (relating to control) preferentially activate the left hemisphere, while reactive coping behaviours (relating to appraisal) preferentially activate the right hemisphere (Tops *et al.*, 2017). Individuals may also exhibit hemispheric dominance reflecting their coping style (Tops *et al.*, 2017). It is therefore possible that this finding has functional relevance. Morphological quantification of numbers of proliferating cells in the HF from both hemispheres of the same hens, combined with a behavioural measure of reactivity (such as TI), would be required to confirm whether a lateralised relationship between AHN and behavioural strategy indeed exists.

6.5.4 Sensitivity of immature neurons at different stages of development

DCX^+ cells with a bipolar (or fusiform) morphology, associated with migration, co-label more frequently with BrdU at 10 days post-injection, while more multipolar DCX^+ cells co-label at day 30 (Balthazart *et al.*, 2008). It has thus been surmised that the latter cells are more mature, and in the process of settling and extending projections to the surrounding circuitry (Boseret *et al.*, 2007). It was previously observed that around two-thirds of DCX-immunoreactive cells in the adult canary telencephalon displayed a uni- or bi-polar fusiform morphology characteristic of migrating neurons, while the remaining third of cells exhibited a multipolar morphology

(Boseret et al., 2007). This is therefore consistent with the finding of a higher density of bipolar than multipolar cells in serial sections from the HF of focal hens in Chapters 4 and 5.

The estimated effect sizes presented in section 6.3.2 indicate that numbers of multipolar cells are consistently more sensitive to stressful experience than numbers of bipolar cells. Indeed, the difference between hens of good and poor physical condition was specific to multipolar DCX^+ cells, though bipolar cells exhibited a trend towards the same relationship. Previously, the suppressive effect of UCMS on DCX^+ densities in the caudal HF was also specific to multipolar cells (Gualtieri *et al.*, 2019). Though a relationship with KBF severity scores existed for adult-born cells with both multipolar and bipolar morphologies, correlations between individual cell densities and KBF severity scores were stronger for the multipolar cells. Progressive loss of immature neurons through apoptosis occurs throughout the developmental stages of AHN (Kempermann *et al.*, 2004), and there are more $DCX^+/Prox1^+/calretinin^-$ neuronal progenitors than $DCX^+/Prox1^+/calretinin^+$ post-mitotic immature neurons across the septo-temporal axis of the mouse hippocampus (Tanti *et al.*, 2013), indicating that fewer differentiating cells with a determined neuronal fate survive to this later stage. As only a small portion of neuroblasts are ultimately recruited for terminal differentiation and functional integration (Kempermann *et al.*, 2004), the summative effects of conditions which modulate neuronal survival and recruitment are likely to be most evident at this more advanced level.

Indeed, in the hippocampus of rats and mice, certain forms of stress with influences beyond immediate proliferation have been demonstrated to preferentially reduce numbers of more mature neuronal precursors, defined by the expression of different stage-specific markers, or by the varying morphologies of cells expressing the same neurogenic marker. While chronic mild stress did not influence rates of 2-hour proliferation, the survival of $BrdU^+$ cells in the DG (for ~3 weeks) was reduced by 20% (Lee *et al.*, 2006). Another study found a slightly greater effect of six to eight weeks of UCMS on four-week survival of immature neurons, compared to the number of cells proliferating over 24-hours (34% and 27% reductions respectively, in relation to controls) (Surget *et al.*, 2011). Furthermore, peripheral LPS-treatment to induce inflammation reduced numbers of DCX^+ differentiating neurons in the DG, but did not alter numbers of $BrdU^+$ proliferating cells (Wu *et al.*, 2007). These effects may also vary with subregion, as UCMS reduced the density of early post-mitotic neurons in both septal and temporal areas of the hippocampus, but also affected numbers of earlier progenitor cells only in the most temporal division (Tanti *et al.*, 2013). Most similar to the present methodology, certain research in rats and mice has also distinguished between the effects of chronic stress on subtypes of DCX^+ cells, defined by their morphology. In both cases, greater reductions were

observed in the numbers of relatively more mature differentiating neurons, following exposure to chronic social (Van Bokhoven *et al.*, 2011) and intermittent restraint (Castilla-Ortega *et al.*, 2014) stress regimes. These findings are thus analogous to the greater sensitivity of multipolar DCX^+ immature neurons to multiple forms of stress apparent in the present research.

6.5.5 Characteristics of the relationship between AHN and experience

Literature in rats and mice suggest that levels of AHN: 1) quantitatively reflect group differences in experience, and 2) integrate positive and negative experiences that occur simultaneously or sequentially. For example, a combination of chronic neuropathic pain and chronic restraint stress resulted in lower levels of AHN than either experience alone (Romero-Grimaldi *et al.*, 2015). Meanwhile, studies of voluntary exercise in rodents have found a positive linear relationship between the amount of running conducted by an individual mouse and immature cell numbers in the HF (Allen *et al.*, 2001; Rhodes *et al.*, 2003). Furthermore, mice with more DCX^+ cells following EE exhibited concurrently lower durations of immobility during the forced swim test (Llorens-Martín *et al.*, 2007), and $BrdU^+$ cell numbers in the temporal rat DG positively predicted sucrose intake during antidepressant-mediated stress recovery (Jayatissa *et al.*, 2006). These findings demonstrate quantitatively linear relationships between behavioural indicators of affective experience and AHN in the rodent brain. Studies in rats and mice do not appear to have explored whether individual differences in the severity or magnitude of an injury negatively predict indices of AHN. However, the negative relationship between individual KBF severity scores and DCX^+ cell counts found in Chapter 4 indicates that AHN in the avian brain co-varies linearly with the magnitude of stress. The apparent homology of regulatory mechanisms suggests that this statement is likely to also be true for the mammalian brain. Optogenetic stimulation of zero to seven-week-old neurons in the mouse DG has revealed that these cells activate local interneurons, which in turn evoke strong inhibitory input to mature granule neurons (Drew *et al.*, 2016). This inhibition is known to influence mature neurons that are preferentially activated under anxiogenic conditions (Anacker *et al.*, 2018). Moreover, levels of AHN strongly affect the degree of inhibition exerted, as this is increased by housing in an enriched environment, but decreased in line with declining AHN with aging (Drew *et al.*, 2016). Given that numbers of surviving immature neurons quantitatively influence the activity of existing hippocampal neurons, a linear relationship between the magnitude of chronic stress experienced and numbers of adult-born cells may relate to an increasing loss of resilience perpetuated by greater stress.

The studies conducted as part of this research project did not directly explore the impact of sequential positive and negative experiences, or of a combination of experiences compared to either in isolation, on resultant numbers of young neurons. This may be an interesting basis for future experiments. However, the most likely interpretation of the housing system study is that a combination of experiences with opposing valence associated with each system had a compensatory influence on DCX^+ cell numbers at the end of lay. A previous study concluded that an aggregate score of many welfare measures also did not differ significantly between furnished-cage and non-cage systems over several flocks (Rodenburg *et al.*, 2008), while authors have highlighted the unique advantages and limitations of each alternative (e.g. Lay Jr. *et al.*, 2011). In rats and mice, a combination of stress and exercise or sexual activity produces levels of AHN intermediate to either experience in isolation, and comparable to control animals experiencing neither (Nakajima *et al.*, 2010; Kiuchi *et al.*, 2012; Yau *et al.*, 2012; Kim *et al.*, 2013). Given that AHN did reflect the group difference in physical body condition, it is unlikely that the null result regarding alternative housing systems is attributable to insensitivity of the outcome measure. It is therefore likely that levels of AHN in the avian brain also integrate experience of positive and negative valence, so that mixed experiences quantitatively resemble neutral experiences.

6.6 Conclusions

Overall, neurogenesis in the HF of adult laying hens appears to reflect exposure to various forms of chronic stress, and is notably suppressed by severe KBFs and in association with poor physical body condition. Systemic inflammation, arising from dysregulation of HPA-axis activity and immune function, may mediate the relationship between these sources of stress and impaired hippocampal plasticity. The positive relationship between *PCNA* mRNA expression in the rostral HF and time spent in outdoor areas of a free-range system suggests that AHN in laying hens, as in passerine birds, is stimulated by environmental complexity and spatial-cognitive demands. Generally, the findings have highlighted associations of individual differences, in areas including coping style, environmental preferences and susceptibility to ill health and/or chronic psychological stress, with AHN and welfare. Results support homologous modulation of AHN in the avian and mammalian HF by chronic negative stress and cognitive challenge, and provide some further evidence for functional specialization across the longitudinal (septo-temporal) axis of the chicken HF. Overall, AHN appears to be a useful tool for assessing the relative degree of chronic stress experienced by groups of laying hens in particular conditions, and numbers of differentiating immature neurons may be quantitatively sensitive to the magnitude or duration of this experience (at least above a certain threshold for

detectable effects). Whether AHN is a responsive measure of positive welfare in poultry, and thus stimulated by experiences known to induce a positive affective state, may require further validation. Welfare implications of the present findings suggest that the longer term subjective experience of commercial hens may be significantly improved by interventions to reduce the prevalence of KBFs and limit the numbers of individuals exhibiting a poor body condition, for example through measures to restrict the spread of infections and re-direct negative social behaviours such as feather-pecking.

6.7 References

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