Exploration of Inflammation-related markers in Hens experiencing Chronic Stress

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ABSTRACT

Reduced adult hippocampal plasticity in hens has recently been demonstrated to be a consequence of chronic stress. This has been observed in multiple studies measured by reduced cellular density of doublecortin (DCX) neurons in the rostral and caudal hippocampal formation (HF). This includes hens with severe keel bone fractures (KBF), poor physical condition and unpredictable chronic mild stress (UCMS). Chronic stress is known to trigger activation of the hypothalamus pituitary adrenal (HPA) axis and ultimately lead to the rise in secretion of glucocorticoid hormones. However, there was no consistent increase in glucocorticoid hormones across these studies.

This thesis investigates the involvement of inflammation in the periphery and the rostral and caudal HF in birds where chronic stress led to reduced DCX^+ density. As a first step, this thesis measured gene expression of inflammatory cytokines in the spleen and HF where DCX^+ neurons were reduced in birds with severe KBF and poor physical condition. There was no change in expression or any correlation with DCX^+ density. Secondly this thesis measured density of microglia cells which have neuroprotective and neurotoxic characteristics by staining hippocampal sections with microglia marker IBA-1. This was to determine if the phenotype of microglia cells differs between birds with severe and minimal KBF who had reduced DCX^+ density, towards an inflammatory ameboid microglia. The severity of the KBF does not lead to morphological changes between ameboid, rod and ramified microglia.

Finally, this thesis utilized UCMS as a method of inducing chronic stress in a time-controlled manner to determine if inflammatory cytokine expression changes throughout, which in this instance it does not. As there was no evidence of inflammatory cytokines and microglia involvement, astrocytes and their related markers were measured in the HF. This thesis found UCMS led to increased gene expression of astrocyte marker GFAP in the caudal HF and increased complement-3 protein across the rostral and caudal HF. These results suggest chronic stress could lead to increased presence of astrocytes and activation of the complement pathway in the HF. Astrocytes are known to secrete complement proteins which can lead to neuronal dysfunction. This points toward a potential role for astrocytes and the complement system in the HF due to chronic stress in hens.

COVID-19 IMPACT STATEMENT

This PhD began in September 2019 and indented to be a 4.5-year studentship. Unfortunately, 6 months into my PhD the COVID-19 pandemic hit. This resulted in closure of the University in March 2020, where I did not return until September 2020. During this time, due to a change in project two months prior in January, I did not have any data to analyse during these months. When I did return to university and begin to conduct my research, pre-existing plans were changed due to the pandemic. This included collaboration with the University of Liverpool to run an experiment in hens with induced inflammation and Unpredictable Chronic Mild Stress. As social distancing and closures continued into 2021, this experiment was unable to go ahead and resulted in the collaborator eventually leaving post. As a result, this research took advantage of pre-existing samples to conduct primary research, though these were limited. For example, it would have been desirable to quantify other tissues such as spleen tissue at more opportunities.

To compensate for the interruptions during the pandemic, a 6-month BBSRC funded extension was granted. However, this extension was granted in April 2022 meaning alternative research plans had already been conducted.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	**Explanation**
ACTH	Adrenocorticotropic Hormone
AHN	Adult Hippocampal Neurogenesis
ANOVA	Analysis of Variance
AVP	Arginine Vasopressin
AVT	Arginine Vasocotin
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
C. Pusila	Cischweinifia Pusila
C3	Complement Protein 3
CA	Cornu Ammonis
CASP-1	Caspase-1
CMS	Chronic Mild Stress
CNS	Central Nervous System
CRH	Corticotrophin-releasing Hormone
CRHR-1	Corticotrophin-releasing Hormone Receptor 1
CSFR-1	Colony Stimulating Factor Receptor-1
DAB	Diaminobenzidine
DAMP	Damage Associated Molecular Pattern
DB	Dark Brooder
DCX	Doublecortin
DG	Denate Gyrus
EAE	Experimental Autoimmune Encephalomyelitis
EAE	Experimental Encephalomyelitis
EE	Enriched Environment
ELISA	Enzyme Linked Immunosorbent assay
EMMEANS	Estimated Marginal Means
EU	European Union
GFAP	Glial Fibrillary Acidic Protein
GHRH	Growth Hormone-releasing Hormone

GnRH	Gonadotrophin-releasing Hormone
GR	Glucocorticoid Receptor
H/L	Heterophil/Lymphocyte
HF	Hippocampal Formation
HPA axis	Hypothalamic Pituitary Adrenal axis
HVC	High Vocal Centre
HVZ	Hypothalamic Ventricular Zone
IBA-1	Ionized Calcium Binding Adaptor Molecule-1
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IR	Infrared
KBF	Keel Bone Fracture
LB	Lohman Brown
LBR	Lamin B Receptor
LMER	Liner Mixed Effects Model
LPS	Lipopolysaccharide
A. Macularia	Actitis Macularia
MHC	Major Histocompatibility Complex
MR	Mineralocorticoid Receptor
MS	Multiple Sclerosis
NCBI	National Centre for Biotechnology Information
NF-kB	Nuclear Factor kappa-b
NG	Neurogenesis
NLRP3	Nod-like Receptor Protein
nNOS	Neuronal Nitric Oxide Synthesis
NO	Nitric Oxide
NPC	Neural Precursor Cells
NSPC	Neural Stem Progenitor Cells
OCT	Optimal Cutting Temperature Embedding Medium
P2XRR KO	P2X7-Receptor Knockout Mice
PAMP	Pathogen Associated Molecular Pattern

PFA	Phosphate Buffered Saline
PSD-95	Postsynaptic Density Protein-95
PVN	Paraventricular Neurons
RT-PCR	Real-Time Polymerase Chain Reaction
SD	Social Defeat
SVZ	Subventricular Zone
SVZ	Subventricular Zone
TGF	Transforming Growth Factor
TI	Tonic Immobility
TNF	Tumour Necrosis Factor
TRH	Thyrotrophin-releasing Hormone
UCMS	Unpredictable Chronic Mild Stress
VT4R	Vasocotin 4 Receptor
VZ	Ventricular Zone
WA	Water Avoidance
Zi	Z Score

CHAPTER 1: INTRODUCTION

1.1 Welfare concerns regarding laying hens

The well-being and health which is often referred to as welfare of animals including laying hens is a major concern to the consumer and the producer, which has led to many changes in how animals are farmed. In the European Union (EU) many hens were housed individually in the conventional battery cage as a way of increasing egg production until 2012, after a gradual phase out which began in 1999 (Appleby, 2003). The conventional cage came under increased scrutiny due to the confined space within these cages which restricted many natural behaviours including nesting and scratching (Tactacan et al., 2009). This led to farmers adopting various other ways of housing laying hens, including furnished cages, free-range with outdoor access and organic (Bonnefous et al., 2022). However, each housing type comes with its own welfare concerns which has led to increased research into the welfare of hens. This section will discuss the welfare concerns which effect hens from caged and non-caged housing systems which may lead to chronic stress, which can lead to negative effects on the birds mood, well-being and egg production.

1.1.1 Risk of pathogen exposure

According to EU guidelines, free-range housing is a system which houses birds at night, but provides free outdoor access during daytime (Marie-Laure Augère-Granier, 2019). This housing system is generally accepted to allow the bird to perform many of its natural behaviours which are limited in the cage setting, including foraging, stretching and pecking (Rufener, Berezowski, et al., 2018). Despite these benefits, outdoor access can increase the hens' exposure to pathogens and infectious disease (Bonnefous et al., 2022). Outdoor access poses this greater risk due to the inability to avoid contact with infectious agents and inability to disinfect the area. The viral infection *Avian influenza* is a particular concern as risk of contamination is greater outdoors through contact with water, floor or buildings that have been exposed to an infected individual and wild birds (Koch & Elbers, 2006). Alongside other infections including *Salmonella enterica subps* which compromises food safety (Van Hoorebeke et al., 2010) and *Campylobacter hepaticus* which impacts mortality and reduced egg production (Crawshaw, 2019). Farmers use a variety of methods to combat this risk of infection including strengthening the immune system through diet. One way of this is supplementation with omega 3 fatty acids which are considered to have anti-microbial effects (Alhusseiny & El-Beshbishi, 2020) and pro-biotics to improve the gut microbiome (Ricke, 2021). Another method farmers often use to manage infection risk is through early detection by routine tests including detection of antibodies to various diseases. For example, the enzyme-linked immunosorbent (ELISA) assay is often used to detect antibody levels in serum and yolk which correlates with exposure and intensity of the *Ascaridia galli* infection (Sharma et al., 2018). When farmers do detect infection in their livestock, it becomes necessary to avoid further spread which can often lead to the culling of livestock (Association, 2017).

1.1.2 Pecking

One major benefit of the free-range housing system for laying hens is that they are able to express many behaviours including the pecking of other hens, although this does come with risk to other hens and could be problematic. Pecking by one hen to another is more frequent within the free-range environment and can even increase in severity leading to the other hen experiencing pain and feather loss (Iqbal et al., 2020). Feather pecking includes the forceful pulling and consumption of another hen's feathers from the back, cloacal vent and tail. Severe feather pecking is thought to occur in free-range and caged housing, affecting between 40%-48% of layer flocks in Europe (Blokhuis et al., 2007). As this behaviour is common and leads to poor welfare of the hen, there are steps to avoid this. Beak trimming is currently used in France and the UK, although this has been associated with welfare concerns (Schwarzer et al., 2021). One study which investigated severe pecking in birds with and without beak trimming found reduced rates of severe pecking, although this behaviour was still persistent (Schwarzer et al., 2021). What this study did find effective against severe pecking was the use of a winter garden or free-range housing alongside sufficient enrichment. Although this is a complex issue, when used in conjunction with each other this could be more effective than beak trimming which leads to additional pain and stress for the hen (Schwarzer et al., 2021).

1.1.3 Space confinement

Hens who are not housed within the free-range system are typically housed in enriched colony cages which is typically up to one third of all layer hens in the UK (Kenneally, 2023). Each enriched cage typically has space for between 40-80 hens and has some horizontal space for natural behaviours including nesting and perching, but not wing flapping or flying (Lay et al., 2011). This means in many cases these hens are confined to smaller spaces without outdoor access. These cages do not contain comparable foraging space to the free-range system, but hens are motivated to forage through the addition of litter (Lay et al., 2011). An enriched cage is considered an improvement from battery cages with the addition of a small nesting area of 25cm^2 per hen, 3”-4” perches that run along the cage, improved height and a small scratching area (Kiess et al., 2012). These guidelines were set by the European Directive (1999/74/EC) in 1999 which legislated to ensure all EU member states complied with the set guidelines for all newly build or rebuilt systems of production which were brought into use for the first time. This included the use of floor installation supporting forward-facing claws, headroom of 45cm between levels, 15cm of perching area per hen and at least one nest for every 7 hens (The Council of the European Union, 1999). Despite these requirements, there are still calls in the British and European parliament to phase out the use of cages with potential legislation being introduced by the EU late 2023 (Ares & Sutherland, 2022; The Council of the European Union, 1999).

An enriched cage provides the hen with an improved environment in comparison to the previously used battery cage. However, despite demand for caged eggs is an increasing demand for farmers to move towards free-range (UK Government, 2023). This is because the use of cages as a whole is believed to have implications for the welfare of hens. One downside of the enriched caged housing system is the lack of space in comparison to free-range housing, where hens in cages have limited space to explore their natural behaviours. When the battery cage ban was announced and the use of enriched cages was introduced, this came with improved horizontal room for the hens (Lay et al., 2011). The increase in horizontal space within the cage allows the hen to perch, nest, forage and dust bathe. However, other behaviours including wing flapping are more restricted (Appleby et al., 2002). It is believed that this space restriction is important for consumers and can also lead to various other concerns including overcrowding and pecking (Rodriguez-Aurrekoetxea & Estevez, 2016).

1.1.4 Enrichment within cages

The enriched cage housing system includes the use of furnishing including perches and nesting, which similarly to the free-range system can lead to fractures of the keel bone (Rufener, Baur, et al., 2018; Sandilands et al., 2009). However, one other concern which may arise regarding hens in cages is where a hen chooses to lay its eggs. Some might give preference to laying whilst crouching on the perch, which many hens are motivated to use (Olsson & Keeling, 2005). A consequence of this is the exposure of the hen’s cloaca whilst laying eggs which could lead to cloacal cannibalism. This is a common concern amongst caged housing and can even lead to death, as it is one of the most common forms of cannibalism (Tablante et al., 2000). Cloacal pecking often occurs when the hen passes a large egg leading to tearing or prolapse of the vaginal mucosa, which leaves membranes exposed to other hens. Pecking of this exposed area often leads to trauma and prolapse of the oviduct (Tablante et al., 2000). Beak trimming which as mentioned is a technique used to reduce pecking in birds sometimes used as a preventative measure for all types of pecking, including feather and cloaca. However, this practice is not routinely used due to be pain during this procedure (Schwarzer et al., 2021).

There is litter provided throughout the enriched cage housing system which allows the hens to dust bathe and provides additional space for the hens to gather (Campbell et al., 2016). Although one problem which arises from this includes the potential overcrowding, as hens are highly ritual in when they bathe (Campbell et al., 2016). One study found that although hens dust bathe throughout the day, there was a peak time for bathing within two flocks, in the afternoon or early morning. During these peak times there was observations of crowding within the litter (Campbell et al., 2016). Crowding of hens could lead to restricted access for others alongside some dominating this space.

1.1.5 Keel bone fractures (KBF)

Hens are also at risk of non-infectious welfare risks from all housing settings. This includes increased risk of bone fractures due to their high activity level and ability to collide with other birds and furniture within the housing system (Casey-Trott et al., 2017). It is considered that the keel bone fracture (KBF) which is often caused by hens colliding with furniture, for example a perch (Nasr et al., 2012; Bonnefous et al., 2022; Rufener, Baur, et al., 2018). Although one author suggested KBF might not always be caused by colliding as hens within caged systems without the ability to move freely also have fractures (Thøfner et al., 2021). One study by Thofner et al 2021 in Danish layer hens compared KBF in non-caged hens with hens from caged systems. That study found fractures were prevalent in all hens affecting between 53%-100% of birds from non-caged housing and 50%-95% from furnished cages, mostly affecting the distal part of the keel bone (Thøfner et al., 2021). KBF are extremely common and one risk factor associated with fractures is impaired mineralization of bones, which could be caused by egg formation requiring a high use of calcium (Gautron et al., 2021). In addition to hens requiring large amounts of calcium for egg production, low body weight has also been associated with increased risk of bone fracture. Hens who had higher prevalence of KBF were often found to be underweight with increased laying performances (Bonnefous et al., 2022; Gautron et al., 2021; Thøfner et al., 2021). One solution that is often used is dietary supplementation with omega-3 polyunsaturated fatty acids, which as previously mentioned is often used for its antimicrobial properties (Alhusseiny & El-Beshbishi, 2020). This dietary addition is thought to be useful for improving bone strength and even reduced fractures by 27% in one study using free-range hens (Toscano et al., 2015). Omega-3 fatty acids are thought to improve bone strength through their ability to form osteoblasts and modulate calcium metabolism. Although there is uncertainty around the economic viability of this use and further research is needed to fully understand the effects supplementation has on bone health (Toscano et al., 2015).

1.1.6 Piling and Smothering

Hens within barns often gather into larger groups which is commonly referred to as piling, which can sometimes lead to mortality. When mortality does arise due to hens piling, this is referred to as smothering (Gray et al., 2020). There might be several motivations for hens to pile which includes some natural behaviours and motivations such as dust bathing and social influences (Gray et al., 2020). It is believed some common welfare concerns discussed earlier including KBF and cloacal pecking are adverse consequences of piling (Gray et al., 2020; Tablante et al., 2000).

1.1.7 Welfare concerns and chronic stress

Despite these varying welfare challenges hens face, some including keel bone fractures have been associated with chronic stress and the physiological mechanisms of this are not clear. There have been some recent studies which associated welfare concerns with chronic stress, including keel bone fracture severity and poor physical condition which included reduced feather coverage (Armstrong et al., 2020, 2022). Both studies observed the reduction of neuronal plasticity in the hippocampus of birds who had severe KBF compared to minimal, alongside birds in poorer physical condition to good condition (Armstrong et al., 2020, 2022). To determine if chronic stress of the hen is driving the reduction of neuronal plasticity, experimental models of chronic stress have additionally found that unpredictable chronic mild stress (UCMS) leads to this same reduction of neuronal plasticity in the hippocampus (Gualtieri et al., 2019). These three studies used doublecortin (DCX) as a marker to measure the density of newly generated neurons across the rostral and caudal hippocampus. DCX is a commonly used marker as it is present during the formation of new neurons during the proliferative stages (Balthazart & Ball, 2015). They found significant reductions in both multipolar and bipolar DCX^+ density (Armstrong et al., 2020; Gualtieri et al., 2019; Armstrong et al., 2022). This review will first set out the stress response before discussing these effects on the hippocampal formation (HF).

1.2 The hypothalamic pituitary adrenal (HPA) axis

1.2.1 Activation of the HPA axis

The HPA axis is a neuroendocrine system which consists of the hypothalamus, pituitary and adrenal glands which develops during foetal life (Sheng et al., 2021). The HPA axis is a major part of the physiological response to stress and consists of three main structures (Smulders, 2021) (figure 1). The first structure of axis is the hypothalamus, a structure within the ventral brain of mammals (Sheng et al., 2021). In the avian species, the hypothalamus can be situated in the anterior and ventral position to the mid brain (Pusch et al., 2023). In the hypothalamus, when stress is first encountered the release of CRH and arginine vasopressin (AVP) in mammals which is known as arginine vasocotin (AVT) in birds is secreted by paraventricular neurons (PVN) (Herman et al., 2016). Secondly in the pituitary gland CRH and AVP/AVT stimulate adrenocorticotropic hormone (ACTH) into the blood stream towards the final structure of the axis the adrenal glands. When ACTH reaches the adrenal glands which are located beside both kidneys, it then stimulates the secretion of the glucocorticoid hormone corticosterone (CORT) in rodents and avian and cortisol in various mammals. This process is orchestrated through a negative feedback loop where increased CORT levels circulating the blood stream will signify a pause in CRH and AVP/AVT secretion in the hypothalamus (Kino, 2015). The negative feedback loop works through the binding of glucocorticoid hormones including CORT which bind to MR and GR receptors present in various parts of the brain. One region of the brain which is rich in these receptors, MR in particular, is the hippocampus. The binding of glucocorticoids to these receptors is reported to have implications on neuronal plasticity and behaviour adaption which has been demonstrated in a mouse model of acute stress. That study found MR and GR receptors regulate many genes including neuron expressing neuronal NOS (nNOS) which is involved with synaptic plasticity (Mifsud et al., 2021).

Increased CORT secretion in the blood stream has been associated with stress in several studies. This includes one study which used food restriction as a chronic stressor in Broiler chickens, found chronically elevated plasma levels of corticosterone (Robertson et al., 2017). Another study discovered Bovan Brown layer hens housed in conventional caged housing had elevated feather corticosterone and spent longer in Tonic Immobility (TI) which is a common method of measuring stress (Campbell et al., 2022). These findings by Cambell and colleagues 2022 suggest caged housing for layer hens might be a stressful environment, leading to elevated CORT levels. There are varying effects of increased CORT on the immune system and brain which will be discussed later in this review along with the HF.

1.2.2 The HPA axis and the immune system

The HPA axis interacts with cytokines including IL-6, IL-1, and tumour necrosis factor (TNF) (Cain & Cidlowski, 2017). For example, one study in hens which administered CORT through drinking water had increased mRNA gene expression of pro-inflammatory cytokines IL-6 and transforming growth factor (TGF)-β in the bursa of Fabricius (Zaytsoff et al., 2019). Zaytsoff et al 2019 did not explore inflammation further focusing on the effects of CORT on water-soluble metabolite profiles in the liver and kidney tissue which were dysregulated. In addition, the study by Robertson et al 2017 which as previously mentioned found increased CORT levels in broiler chickens on a food restricted diet, too had increased spleen gene expression of IL-6 (Robertson et al., 2017). Multiple cytokines are involved with the regulation of the HPA axis. One study suggests anti-inflammatory cytokine IL-10 which is produced in the pituitary and hypothalamic tissue, enhances production of CRH (Smith et al., 1999), which in turn may lead to CORT secretion. As part of the negative feedback loop, secreted glucocorticoids including CORT have further interactions with the immune system. They then bind to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) on the surface of immune cells including monocytes which leads to inhibition of cytokines including IL-6 (Tian et al., 2014). GR and MR receptors are not only present on the surface of immune cells, they are also present on cells that exist within the HF, including microglia. However, during periods of chronic stress HPA axis activity becomes dysregulated through increased CORT secretion and inflammatory cytokines. This has been shown in the avian species where in studies previously mentioned in this chapter where hens administered CORT through drinking water show increased gene expression of splenic IL-6 and feed restricted broilers exhibit elevated CORT levels (Robertson et al., 2017; Zaytsoff et al., 2019).

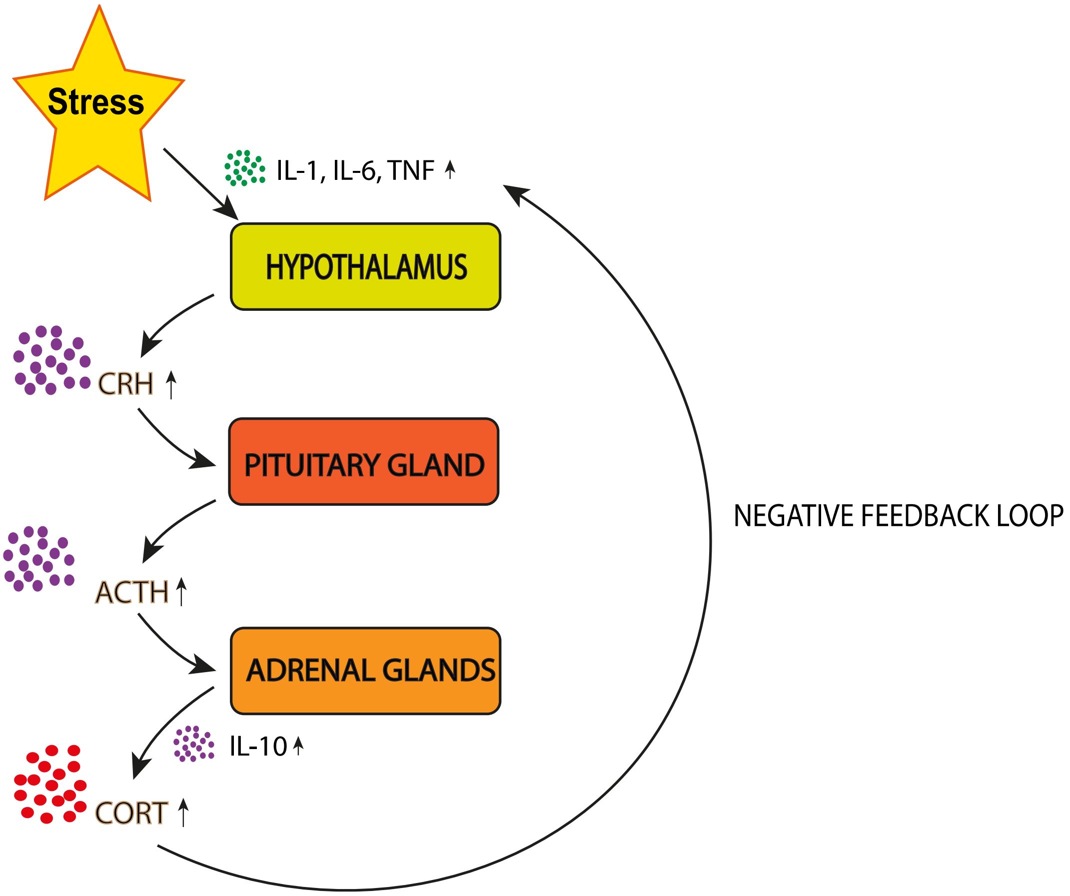

Figure 1: Stress-induced activation of the HPA axis and illustration of the hormones produced and inflammatory cytokines involved.

1.3 Heterophil and Lymphocyte response and CORT

As chronic stress is known to increase CORT levels, this has been associated with a decrease in antibody responses by reducing the number of antibody producing mature B cells as part of the immune response (McGregor et al., 2016). As a result chronic stress in birds is often measured by comparing the ratio of inflammatory cells. (Cotter, 2015). This is known as the heterophil and lymphocyte ratio (H/L). Heterophils are part of our innate immune system, whereas lymphocytes are both innate and adaptive immune cells (Cotter, 2015). Innate immunity is the first line of defence for the immune system which is general and non-specific, whereas adaptive immunity is the immune response following exposure to a specific antigen or pathogen following exposure (Rose, 1974). This technique is commonly used to measure stress amongst birds including hens, through blood from their wing veins. It is suspected that this measurement is easily utilised and gives a measurement of adrenal-corticoid hyperactivity.

Despite this being a commonly used indication of chronic stress and HPA axis activity, one experimental study using UCMS in hens found no difference between H/L ratios between UCMS and the control group (Gualtieri et al., 2019). In addition, the birds with UCMS treatment had lower baseline plasma CORT levels and lower spleen mass suggesting UCMS did not increase CORT levels (Gualtieri et al., 2019). However, what this study did find along with other studies from this same author was UCMS, KBF and poor physical condition which are all potential stressors lead to reduced DCX$^+$ cell density in the hippocampus (Gualtieri et al., 2019; Armstrong et al., 2020, 2022). Collectively these findings by the above studies which show welfare concerns such as KBF and poor physical condition in hens reduce neuron density in the absence of increased CORT secretion point toward the possibility of other physiological mechanisms responding to chronic stress in birds.

1.4 The hippocampal formation and neurogenesis

1.4.1 The Hippocampal formation (HF)

The HF exists within the temporal lobe of the primate brain and is vulnerable to damage by external stimuli, including stress. The avian HF is considered homologous to the mammalian HF for reasons including its role in learning and spatial processing (Székely, 1999). One key difference between the avian and mammalian HF is their respective anatomy.

The mammalian HF consists of the dentate gyrus and the Cornu ammonis (CA) including CA1, CA2, CA3 and CA4 which are both separated by a shallow furrow whilst curving into one another (Anand & Dhikav, 2012). The mammalian HF resembles a curved Seahorse like structure or can often be referred to as the Ammons Horn, whilst the avian HF is a more uniform homogenous structure located at the medial dorsal edge of the forebrain (Gupta et al., 2012). Within the avian HF, there is a dorso-medial region which is most likely homologous to the CA regions and a dorsal-lateral region believed to be homologous to subiculum and entorhinal cortex, which are areas closely connected to the hippocampus in mammals (Striedter 2016). Below and slightly more rostral is the ventral V-shaped region, most likely corresponding to the mammalian dentate gyrus (Striedter 2016). The caudal region of the avian HF is believed to be homologous to the rodent ventral region, alongside the avian rostral corresponding to the mammalian dorsal end (Fanselow & Dong, 2010; Smulders, 2017). The caudal/ventral region of the HF is involved with the regulation of emotions, including stress and anxiety (Gualtieri et al., 2019). The rostral/dorsal region is involved with the main cognitive functions including learning and memory (Fanselow & Dong, 2010). Reduced neuronal plasticity or DCX$^+$ cells in the HF which will be discussed in the next section is a common consequence of chronic stress and poor welfare in hens (Armstrong et al., 2020; Gualtieri et al., 2019; Armstrong et al., 2022; Robertson et al., 2017).

1.4.2 Adult Hippocampal Neurogenesis (AHN)

There is a consistent turnover of new neurons in the HF throughout adulthood to maintain proper functioning which is modulated by external factors including the negative impact of stress and beneficial effects of exercise (Gualtieri et al., 2019; Pérez-Domínguez et al., 2017). The turnover of neurons is essential for the proper functioning of the HF and is compromised by stress in Broiler chickens and hens of the avian species in a similar way to mammals (Gualtieri et al., 2019; Armstrong et al., 2022; Robertson et al., 2017).

It was once thought that the production of new neurons in the HF was restricted to embryonic development (Altman, 1962). This was generally accepted till Altman 1962 measured proliferation of glial cells in the brains of adult rats to find the nuclei of select neurons and neuroblasts were labelled with the proliferation marker thymidine^{-H3}. It was believed these cells had proliferated from previous undifferentiated cells during the period the proliferation marker was circulating as adult neurons to not undergo mitotic division themselves.

Further investigation in adult rats discovered proliferating granule cells in the dentate gyrus (DG) region of the HF measured by ^{3}H-thymidine nuclei incorporation (Altman, 1963). This phenomenon is similar in birds, where widespread neurogenesis occurs continuously throughout the adult brain (Alvarez-buylla & Nottebohm, 1988) (Barnea & Pravosudov, 2012). One study in Zebra Finches found neurons are produced throughout post-hatching life, although proliferation occurs at a higher rate in juveniles compared to adults (DeWulf & Bottjer, 2002). The ongoing production of new neurons is thought to take place to replenish old neurons and also aid learning and memory. One study in male canaries studying mature neurons within the high vocal centre (HVC) which is necessary for the production of the bird song, found neurons die as testosterone levels decrease at the end of the season (Kirn et al., 1994). Neurogenesis has been widely studied in songbirds (Balthazart et al., 2008; Alvarez-Borda & Nottebohm, 2002). It is thought that neurogenesis in the ventricular zone (VZ) is necessary for songbirds to aid the memory of new songs, with new neuron incorporation at a higher rate in the autumn when song changes are more frequent (Brenowitz & Larson, 2015). There has been increased rates of cells positive for thymidine^{-H3} in male Canaries within the HVC which correlated with the total song produced (Alvarez-Borda & Nottebohm, 2002), suggesting neurogenesis aids song memory.

1.4.3 The stages of AHN

Neurogenesis within the HF is a multi-step continuous process throughout life in mammals and birds (Storer et al., 2018; Gualtieri et al., 2019; Garza et al., 2008). In mammals the generation of new neurons in the HF which is also known as adult hippocampal neurogenesis (AHN) comes from adult neural stem/progenitor cells (NSPCs) to give rise to granule cells (figure 2). Eventually these newly formed neurons will reside in the subventricular zone (SVZ) and the dentate gyrus (DG) (Braun & Jessberger, 2014). There are four steps to this process which include: the precursor cell phase, the early cell survival phase, the postmitotic maturation phase and the late survival phase (Kempermann et al., 2015).

The precursor cell phase begins with a pool of cells with radial glia-like properties with their cell body present in the SGZ and processes extending towards the molecular layer which could eventually become neurons. These precursor cells show signs of self-renewal and potency and appear as highly proliferative cells eventually becoming a type two neuronal progenitor cell (Kempermann et al., 2015). It is at this point DCX is expressed which has been widely used as a marker of AHN (Troubat et al., 2021; Gualtieri et al., 2019; Armstrong et al., 2022). However, the use of DCX as a marker of neurogenesis will be discussed in section 1.6.4.

The next phase of AHN is the early survival phase where the neural progenitor cells exit the cell-cycle and become neuroblasts. They then enter the postmitotic stage where progenitor cells become immature neurons with axonal and dendritic outgrowth.

Finally, the late survival phase begins with the mature neurons that successfully formed connections and maturity become integrated to the existing circuit (Kozareva et al., 2019).This process has been demonstrated in the adult avian brain through cellular proliferation marker thymidine^{-H3} (Alvarez-buylla & Nottebohm, 1988). This study found that when they used thymidine injection there was a 68% increase in elongated cells in the ventricular zone (VZ) which increased by a further 63% by day 3 assuming division. By day 20 these cells gradually decreased by 5.2% and a further 3.5% decrease by day 30 as cells began to migrate. A new migrating cell was soon discovered with elongated nuclei alongside two darkly stained nucleoli close to the lateral ventricle. These cells had increased in number and moved laterally clustering in two cohorts by day 6 parallel to radial glia fibres (Alvarez-buylla & Nottebohm, 1988). That study concluded the increase in those migrating cells on day 6 was equal to the decrease in cells at the VZ. Neurogenesis is thought to occur to a number of avian brain regions including the paraolfactory and parahippocampal region (Barnea & Pravosudov, 2012).

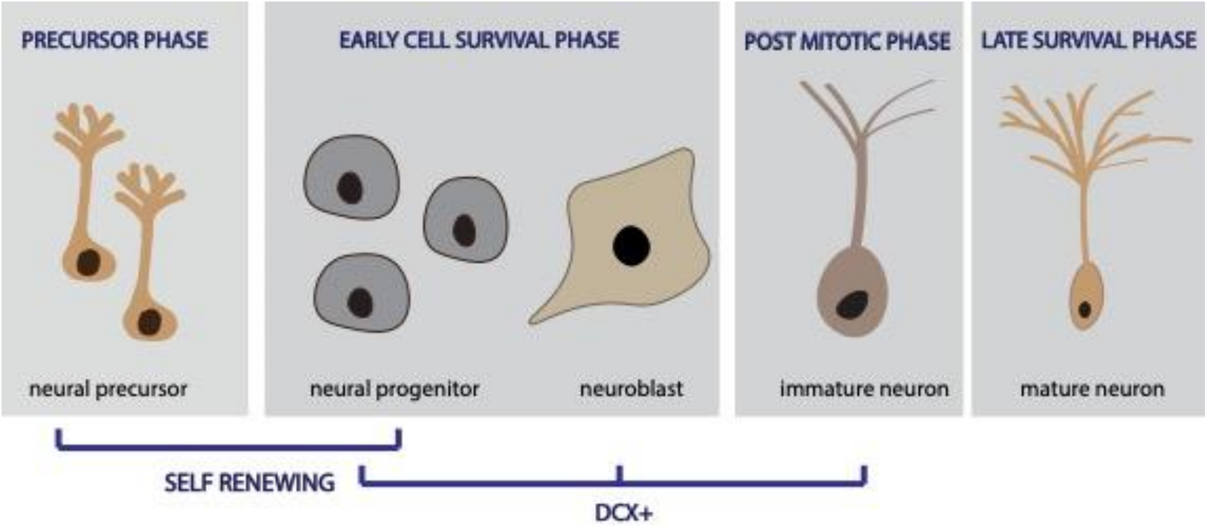

Figure 2: The 4 stages of adult neurogenesis and their respective cellular development from neural precursor cells to a mature neuron

1.4.4 Doublecortin as a marker of neurogenesis

In both avian and mammalian species, DCX has been frequently used as a marker of neurogenesis. This use includes prior studies in broiler chickens and layer hens to link chronic stress to the reduction of AHN, where reduced DCX^{+} expression was used as an indication of reduced neurogenesis (Armstrong et al., 2020; Gualtieri et al., 2019; Armstrong et al., 2022; Robertson et al., 2017). DCX is a neuronal migration protein which is required for the normal migration of neurons into the cerebral cortex (Balthazart & Ball, 2014). DCX is typically expressed in immature granule cells which are beginning to migrate, but expression is no longer present once these cells become fully mature neurons (Spampanato et al., 2012). It is believed the expression of DCX lasts for 30-40 days in the HF of mammals beginning in late mitotic neuronal precursors up until they are post-mitotic neurons, however, this may differ in other species (Balthazart & Ball, 2015). Recent evidence suggests DCX expression is not limited to migrating neurons and may not be a reliable marker of neurogenesis in canaries and other songbirds like it is in mammals. In the adult canary brain, DCX$^+$ neurons have been observed in the telencephalon which is a neurogenic area of the brain, although this study did not give any reference to the HF (Vellema et al., 2014; Balthazart & Ball, 2015). However, DCX expression was not limited to neurogenic regions and expression was observed in regions where neurogenesis does not occur including the ventral tegmental area of canaries (Vellema et al., 2014; Balthazart & Ball, 2015). A similar phenomenon was observed in mammals prior to this, where DCX$^+$ cells were found in non-neurogenic areas of the brain including the piriform cortex of adult mice (Klempin et al., 2011). That same study compared this DCX$^+$ population of cells with the DG of the HF, which is considered neurogenic. These authors compared the proliferative activity of the DCX$^+$ cells in the piriform cortex and found these cells were post-mitotic, suggesting these cells were not neuroblasts as they exist in the DG (Klempin et al., 2011). Furthermore, the population of cells in the piriform cortex had large Na$^+$ currents and multiple action potentials which would be low in the DG (Balthazart et al., 2008). This author concluded that DCX is a suitable marker of neuronal plasticity, rather than neurogenesis (Balthazart et al., 2008).

In birds, studies have been carried out by double staining for DCX and BrdU which marks proliferating cells. Adult male canaries were injected with BrdU for 5 consecutive days then their brains were collected 30 days later. This study took HVC sections and double stained for DCX and BrdU, detecting large numbers of double stained cells, with 70% of all DCX$^+$ cells additionally BrdU$^+$ on day 10 (Balthazart et al., 2008). As BrdU is present for DNA incorporation after injection, it was determined these cells represented new neurons. As this study continued from days 10-30, BrdU$^+$ cell numbers decreased when DCX$^+$ remained consistent with no decrease. This indicated DCX expression lasts long after cells become post-mitotic (Balthazart et al., 2008). DCX is still used but as a marker of neuronal plasticity rather than neurogenesis in birds (Armstrong et al., 2022), which this phenomenon is typically supported by other authors (Balthazart & Ball, 2015; Vellema et al., 2014).

1.5 Interactions between the hippocampal formation and the HPA axis in mammals and birds

1.5.1 In mammals

In mammals, the consistent development of new neurons in the HF in adulthood is susceptible to stress and involves the HPA axis (Kino, 2015). This process and its importance will be discussed in later sections. The HF in particular is considered to be particularly susceptible to stress where stress-led release of corticosteroids from the hypothalamic-pituitary-adrenal (HPA) axis are a mediator of the effect of stress on the hippocampus (Kim et al., 2015). There has been an abundance of studies investigating chronic stress exposure with elevated CORT levels resulting to effects on hippocampal morphology. However, many of these studies were in mammalian species. The increase of CORT has always been a likely mechanism to drive adult hippocampal neurogenesis (AHN) or neuronal plasticity reduction due to the abundance of type-1 MR and type-2 GR receptors in the hippocampus which as previously mentioned are regulated back to the HPA axis via a negative feedback loop (Kim et al., 2015). Experimental exposure to CORT via injection or water to rats revealed reduced AHN and the onset of hippocampus-dependent symptoms including anxiety- and depression-like behaviours (Kim et al., 2015). The mechanisms that lead to reduced AHN are more defined in mammals than birds. Mammalian research indicates consistent stress can lead to the suppression of new neurons being produced, which has also been correlated with increased glucocorticoid levels (Gould & Tanapat, 1999). A study which used social defeat (SD) as a stressor hypothesised that in mice without neurogenesis (NG - or +) glucocorticoids secreted during periods of chronic stress led to depressive-like behaviour. Therefore they suggest if glucocorticoid levels were clamped in NG^{-} mice, there would be a prevention in stress-induced neurogenesis decrease and confer resilience to SD (Lehmann et al., 2013). This study showed that during SD, glucocorticoid secretion is required for the reduction of AHN in SD mice when the NG^{--} group did not show signs of depressive-like behaviour (Lehmann et al., 2013). However, this same study provided an enriched environment (EE) known to increase neurogenesis for one group post SD to conclude both SD and EE stimulate the HPA axis, but with opposing effects on cell survival. It is likely that a moderate elevation of CORT might favour a hippocampal-dependant memory consolidation and spatial memory foundation. Alternatively, a robust increase might impair AHN survival (Zhu et al., 2014). What this study does indicate is that neurogenesis is involved with stress resilience and that increased CORT with decreased neurogenesis during SD could be permissive to a secondary set of stress-induced mechanism resulting in depressive like behaviour.

1.5.2 The effects of chronic stress in the avian HF

Like mammals, avian AHN may be responsive to stressors. Recent emerging evidence suggests decreased levels of AHN in the caudal hippocampus of hens may be linked to stress caused by poor physical condition, KBF and UCMS (Armstrong et al., 2022; Gualtieri et al., 2019; Armstrong et al., 2020).

The latest study by (Armstrong et al., 2022) explored AHN plasticity in birds from two generally used housing systems: multi-tier and colony, which were the commercially used housing systems at that farm. Birds of good and poor physical condition were selected by (Armstrong et al., 2022) to find lower multipolar neurons expressing DCX$^+$ across the HF in birds of poor body condition, from both housing systems. Furthermore, this same study found gene expression of pro-inflammatory cytokine interleukin-6 (IL-6) to be increased in the spleen of all birds of poor condition. This same research group further found birds subject to UCMS featured less multipolar neurons (DCX$^+$) within the caudal pole of the HF (Gualtieri *et al*., 2019). In the final study cited, Armstrong et al 2020 found a similar effect in hens with severe KBF, which had lower densities of DCX$^+$ multipolar and bipolar neurons present in the hippocampus which can indicate birds with fractures experienced more pain and stress leading to this reduction.

Despite many studies suggesting increased glucocorticoid hormones leading to various effects on the hippocampus, birds with UCMS treatment had lower plasma baseline CORT levels alongside reduced AHN in the caudal hippocampus (Gualtieri *et al.*, 2019). With this conclusion and others including (Kim et al., 2015; Lehmann et al., 2013) hint that whilst elevated HPA axis hormones are highly susceptible to stress, they are involved with various other metabolic processes and do not respond uniquely to stress (Koolhaas et al., 2011). Ultimately, this might suggest glucocorticoid secretion may only be a small fragment and not solely responsible in mediating reduced AHN in the caudal pole following UCMS exposure (Lehmann et al., 2013; Kim et al., 2015).

1.5.3 The role of inflammatory cytokines in stress-induced reduction of immature neurons

This leaves us with the question of if not only the HPA axis, how does chronic stress lead to a reduction in neuron formation? Many non-exclusive pathways can be involved with activation of the HPA axis including the immune system. The immune system and HPA axis are in constant contact through the secretion of cytokines known as interleukins (IL) and tumour necrosis factor (TNF) which can activate the HPA axis. Inflammatory cytokine IL-1 is considered the most potent when activating the HPA axis (Bellavance & Rivest, 2014). Where chronic stress can lead to activation of the HPA axis and increased glucocorticoid secretion, in turn the expression of cytokines changes. Here inflammatory cytokines which initially activated the HPA axis were secreted are now downregulated where we then see increased secretion of anti-inflammatory cytokines including IL-10 (Tian *et al*., 2014). Additionally, during local inflammation in the brain, inflammatory mediators like cytokines may trigger activation of the HPA axis which was previously discussed in section 1.2.2. When frequently exposed to glucocorticoids secreted by the HPA axis, neurodegeneration can occur (Odaka *et al.*, 2017). For example, neurodegenerative conditions including stroke negatively impact adult neurogenesis. The proinflammatory cytokine IL-1 is secreted during episodes of inflammation and when bound to receptor IL-1R1 on neuronal cells, this leads to the expression of further inflammatory cytokines through activation of the inflammasome (Wu *et al.*, 2013).

Additionally, mammalian hippocampal neural stem cells express IL-1R1 and become apoptotic when bound with IL-1β (Wu *et al.*, 2013). We are yet to discover if this is homologous in the avian hippocampus, as it is not clear what role the avian immune system plays in mediating the effects of chronic stress on the reduction of new neurons. Although gene expression of IL-6 has been increased in the spleen of birds with reduced neuronal plasticity measured by a reduction of DCX$^+$ neurons when hens were in poor physical condition, which includes poor feather coverage and a pale comb (Armstrong et al., 2022). It is not clear if this increase is directly involved with activation of the HPA axis, as CORT levels were not measured in that study. However, when CORT was measured in hens with UCMS treatment, the UCMS group had lower baseline CORT levels than the controls (Gualtieri et al., 2019). A further study this time in broiler chickens found their method of chronic stress which was feed restriction increased CORT levels in blood plasma which correlated with the reduction of immature neurons (Robertson et al., 2017). Similarly to the hens in poor physical condition which includes reduced feather coverage and red eyes (Armstrong et al., 2022), this study also found increased IL-6 gene expression in the spleen of birds with feed restriction (Robertson et al., 2017). What these studies do indicate is that CORT is not always increased during episodes of chronic stress in the avian species, but these studies do find reduced neuronal plasticity in the HF (Armstrong et al., 2022; Robertson et al., 2017; Gualtieri et al., 2019). So far there is some evidence to suggest that inflammation might be involved with chronic stress and reduced DCX$^+$ neurons in the HF by increased IL-6 spleen expression in broiler chickens and hens (Robertson et al., 2017; Armstrong et al., 2022). Nonetheless, none of these studies measured expression of a full profile of inflammatory and anti-inflammatory cytokines in the blood, spleen and the HF. It remains unclear if cytokines are involved with the reduction of DCX$^+$ neurons in the HF in hens during chronic stress.

1.6 Neuroinflammation

1.6.1 The activation and function of microglia

The brain can be a local producer of inflammation or respond to systemic inflammation during chronic stress independently of the HPA axis via inflammatory cytokines and brain resident immune cells known as microglia (Walker & Spencer, 2018). Alternatively, circulating cytokines might trigger the activation of microglia which are macrophages exclusive to the brain (Reemst et al., 2016). Microglia cells have both neuroprotective and neurotoxic roles and are found within the HF (Greene & Pisano, 2012b). Under normal circumstances microglia scout the environment to maintain neuronal health. They might do this by secreting anti-inflammatory cytokine IL-10 and brain derived neurotrophic factor (BDNF) (Miranda et al., 2019). In the event of a neuronal injury microglia then release inflammatory cytokines including IL-1β and IL-6 to trigger an inflammatory cascade. One study injected LPS into the periphery, which is a potent activator of the immune system, including secretion of inflammatory cytokines into adult female rats. This study found a strong up-regulation of inflammatory cytokines and a 240% increase in the density of microglia in the dentate gyrus (Monje et al., 2003). Under inflammatory circumstances microglia are highly influential in neuronal survival and can facilitate apoptosis of nearby cells when exposed to tissue damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) (Pérez-Domínguez et al., 2017). Microglia are also able to phagocytose nearby cells and secrete of nitric oxide (NO) (Guadagno et al., 2015; Biscaro et al., 2012) in the presence of IL-1β and IL-6 in the local environment. For example, inducible nitric oxide synthase (iNOS), which produces NO, is expressed by microglia. A study which used an adult quail model found that LPS treatment which is associated with increased inflammatory cytokine expression can induce the overactivation of microglia and lead to the significant upregulation of iNOS (Yang et al., 2011). Despite there being a wide range of research in the mammalian literature, little has yet been discovered linking inflammation as part of the stress-led reduction in AHN in avian species, but more importantly hens.

Microglia are versatile cells which their function is determined by the environment it scouts, which is also reflected in the cells' morphology (figure 3). For example, when a microglia cell is active under non-inflammatory conditions and no brain injury for example stress is present, this is known as the ramified morphology or M1 type microglia. This is where the microglia have elongated and branched processes which project from the cell body and typically produce anti-inflammatory markers, such as IL-10 (Guo et al., 2022). In the presence of DAMPs and inflammatory cytokines including IL-1, microglia retract their processes and switch to an ameboid phenotype with no processes and an enlarged cellular body (Jurga et al., 2020).

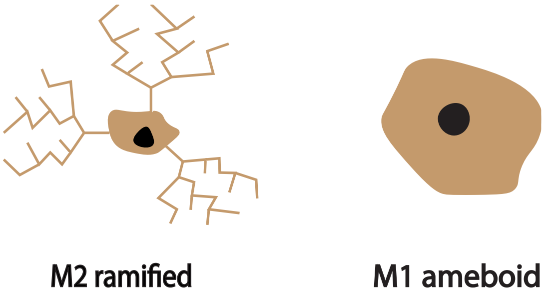

Figure 3: Morphology of M2 ramified microglia with elongated processes and a M1 ameboid microglia with retracted processes and an enlarged cell body.

1.6.2 Microglia in the hippocampus

There is emerging evidence within the avian literature that glial cells including microglia have an important role in the HF. One study looked at two types of Sandpipers: *Actitis macularia* and *Cischweinifia pusila* and stained for microglia and neurons within the HF (Diniz et al., 2016). It was thought that because each sandpiper took different autumn migration routes the hippocampal volume, hippocampal neurons and glial numbers would differ between the two. Although there were no differences in the total of hippocampal neurons which was the case in the study by Armstrong and colleagues 2022 where there were differences in hippocampal volume and glial cells. The *C. pusila* sandpipers had an overall larger hippocampal volume and many more glial cells, suggesting an important role for microglia in birds which might not be directly related to the total number of hippocampal neurons (Diniz et al., 2016). A further study which looked at food-caching birds in captivity vs living freely found the birds living free with access to the natural environment had greater hippocampal volume and glial cells, but no changes in neurogenesis similarly to the sandpiper study (Roth et al., 2013).

One-way microglia are considered to have an important role within the hippocampus is through BDNF signalling. Microglia have been associated with learning and memory by promotion of learning-related synapse formation via BDNF signalling. The same authors as the sandpiper study (Diniz et al., 2016) demonstrate this possibility where they observe significant correlations between the morphology of microglia within the dentate gyrus and visuospatial learning and memory tasks within the monkey brain (Santos-Filho et al., 2014). Another study which looks at microglia morphology from rodents with lower and cognitive performances in hippocampal dependant tasks are less ramified vs more ramified (Viana et al., 2013).

1.6.3 Microglia and chronic stress

In the event of chronic stress, microglia are able to change their phenotype in response to the release of DAMPs and inflammatory cytokines (Frank et al., 2020). One study in adult rats demonstrated the involvement of microglia in the HF following 12 weeks of chronic mild stress (CMS) (Wang et al., 2018). There was a greater number of Ionized Calcium Binding Adaptor Molecule-1 (IBA-1^+) microglia in the hippocampus of the CMS group, in addition to greater protein levels of IBA-1 and CD11b. IBA-1 is a commonly used marker of microglia and CD11b involved with cellular migration during inflammation (Wang et al., 2018). To determine if these microglia were increased during local inflammation of the hippocampus, this same study measured gene expression of inflammatory cytokines. In the CMS group only, there was an increase of IL-1β, IL-6, IL-18 and anti-inflammatory IL-4 and IL-10 (Wang et al., 2018). The increase in pro-inflammatory cytokines which are associated with ameboid microglia and anti-inflammatory which are associated with ramified microglia suggests CMS leads to an increase in microglia with varying phenotypes (Wang et al., 2018). Consistently with the increase in IBA-1 and inflammatory cytokines during CMS, Wang et al., 2018 found increased gene expression of caspase-1 and NLRP3 which are components of the inflammasome to produce inflammatory cytokines in response to DAMPs (S. Li et al., 2022). As KBF have been demonstrated in the brain of hens to increase gene expression of tumour necrosis factor (TNF)-α, IL-6 and iNOS (Wei et al., 2019), it is of interest if microglia are involved and if this effect is present in the HF. One other study in Japanese Quail report an increase of IL-1β/IL-10 gene expression in the HF during postnatal stress but report no differences in colony stimulating factor receptor-1 (CSFR-1) gene expression, which is a marker for all microglia cells. Therefore, this ultimately indicates no differences in microglia numbers (Walker et al., 2019a). However, that study used CORT as a pre- and post-natal stressor. Therefore, it is consistent with Wei et al., 2019 who report increased inflammatory cytokines and elevated CORT levels during KBF. But this does not exclude the involvement for microglia during local inflammation of the HF during chronic stress of birds in a similar way to the findings in adult rats where inflammatory cytokines and microglia numbers were increased during CMS (Wang et al., 2018). Therefore, it is possible where chronic stress does not increase CORT levels as it did not in the (Gualtieri et al., 2019) UCMS study but DCX$^+$ densities in the HF decreased, there could be involvement of microglia and inflammation.

Microglia in their ameboid state are considered to have a detrimental effect on neurons and could be impacted by chronic stress. Microglia typically maintain homeostasis by phagocytosing excess neurons in the HF, which worsens with inflammatory challenge (Greene & Pisano, 2012b). One mechanism of this is through microglia-derived IL-1β which activates p53, a transcription factor involved with the cell cycle in neural precursor cells (NPC). When once study extracted NPCs from mice and cultured with activated microglia and LPS, they found increased p53 protein levels and reduced neurospheres compared to a naïve stem cell media (Guadagno et al., 2015). The findings in this study suggest reduced proliferation through cell cycle arrest and death by microglia-derived factors, as IL-1β, IL-6 and TNF-a were additionally increased (Guadagno et al., 2015). There is a lack of research into the role microglia and inflammation might have in stress-led reduced neuronal plasticity in birds, which is of interest to this thesis.

1.7 The aims of this thesis

This thesis aims to explore the involvement of the immune system in models of chronic stress in hens where reduced neuronal plasticity in the HF was observed consequently. Recent studies which investigated chronic stressors observed reduced neuronal plasticity or DCX^+ cell density in the rostral and caudal HF of hens, which included commonly used stressors including UCMS and feed restriction (Gualtieri et al., 2019; Robertson et al., 2017). The increase of CORT hormones is a common response to chronic stress, however these studies suggest increased plasma CORT levels does not always occur with chronic stress as it was increased during feed restriction (Robertson et al., 2017), but not UCMS (Gualtieri et al., 2019). Therefore, this thesis aims to explore the involvement of the immune system during the stress response of hens by measuring inflammatory cytokine gene expression, such as IL-1β, which is typically associated with activation of the HPA axis (Tian et al., 2014).

In chapters 2+3 this thesis will measure a larger profile of cytokines including IL-1β, IL-6, TNF-α and anti-inflammatory IL-10 in the spleen and again in the rostral and caudal HF to explore local inflammation. Increased IL-6 gene expression in the spleen of hens has been found when birds are in poor physical condition, which is considered a chronic stressor with reduced neuronal plasticity in the HF (Armstrong et al., 2022). Inflammatory cytokines have been associated with chronic stress through KBF, where increased gene expression of IL-1β and TNF-α was found in the brain (Wei et al., 2019). In addition, KBF have also been found to reduce neuronal plasticity in the HF (Armstrong et al., 2020). However, neither of these studies measured expression of these inflammatory cytokines specifically in the HF or made any association between reduced neuronal plasticity in the HF and increased inflammation-related genes following fracture/chronic stress. With that in mind, this thesis aims to further determine any correlation between the expression of these genes and DCX^+ cells in the HF, to explore any involvement with reduced neuronal plasticity.

Following on from measuring inflammation-related genes such as IL-1 in the spleen and HF, this thesis will take a two-step approach in investigating the involvement of microglia during chronic stress and again compare this to DCX^+ density. As a first step, In chapters 2+3 I plan to measure gene expression of microglia activation marker CSFR-1, to determine if chronic stress leads to changes in active microglia in the HF. Secondly, in chapter 4 this thesis aims to investigate the morphological phenotypes of microglia: M2 ameboid which respond to inflammatory cytokines to enter their neurotoxic state and M1 ramified which circulate the brain under normal circumstances using the IBA-1 antibody which was adapted from mammalian studies for its use in this study.

CHAPTER 2: DOES EXPRESSION OF INFLAMMATORY CYTOKINES DIFFER BETWEEN HOUSING SYSTEMS OF LAYING HENS?

2.1 Abstract

Reduced DCX^+ cells in the hippocampal formation has recently been observed to be a marker of chronic stress in laying hens. It is currently unknown what the physiological mechanisms are behind this stress driven decrease. This chapter will measure gene expression of inflammatory and non-inflammatory cytokines in hens from two housing systems: multi-tier with outdoor access and furnished colony housing were used with birds in good and poor physical condition. In the HF and spleen of birds from colony and multi-tier housing systems genes IL-1β, IL-6, TNF-a alongside anti-inflammatory IL-10 and CSFR-1 and BDNF in the HF only will be measured. A study prior to this chapter with the same birds found the birds in poor condition from both housing system had reduced DCX^+ density in the HF, whereas the study in this chapter will expand upon those previous findings by exploring the potential involvement of inflammatory cytokines. In addition, this present chapter explored a potential correlation between expression of any genes measured and DCX^+ density in the HF. The data presented in this chapter found there was no correlation between any of the genes measured and the DCX^+ density obtained from previous work. However, there was an increase in IL-10 expression in the spleen and HF alongside increase spleen mass of in the birds housed in the colony system. The increase in IL-10 expression and spleen mass could be due to the colony system birds receiving antibiotic treatment prior to analysis for poor laying performance, or because of prior inflammation.

2.2 Introduction

Chronic stress in chickens has recently been associated with the suppression of DCX^+ neurons in the caudal pole of the hippocampal formation (HF) in hens facing unpredictable chronic mild stress (UCMS) (Gualtieri et al., 2019). This is thought to be in a similar fashion to the ventral region in mammals which is sensitive to stress and emotional responses (Troubat et al., 2021; Gould & Tanapat, 1999). A recent study in hens set out to explore whether the different housing systems for hens, including the multi-tier with outdoor access and large furnished colony cages, were a cause of chronic stress. The study measured the density of doublecortin (DCX^+) multipolar and bipolar neurons in the HF as a biomarker for stress. There are a range of housing systems used for laying hens which each come with different welfare challenges, including a conventional cage system, furnished colony cages and the multi-tier system with outdoor access. Conventional cage and furnished colony cage housing is typically a confined environment. Hens are restricted in performing certain behaviours (Lay et al., 2011). Hens often do not fully acclimatize to prolonged special restrictions and prefer more space. When hens are offered larger spaces there is often a display of behaviours including intense wing flapping, tail wagging and stretching. The intensity of these behaviours has been correlated with the time of confinement (Nicol, 1987). Access to food and water might become limited to a proportion of hens due to others blocking access with potential dominant hens intentionally blocking access which could result in other hens feeling threatened (Hughes, 1983). A dense population of hens within a confined setting also increases the risk of feather pecking, smothering and cannibalism (Hansen, 1976). The multi-tier system is sometimes considered to have advantages over the conventional system as the multiple platform offering with nesting and perching at different levels is said to promote environmental exploration and the ability to perform natural behaviours that are restricted in the conventional setting (Lay et al., 2011). Hens are said to prefer perches at a higher level than lower, providing the multi-tier system with an advantage. This does however raise further concerns including increased likelihood of falling from the perch and could lead to keel bone fractures (Wilkins et al., 2004) which itself has been found to lead to AHN reduction and the onset of depressive-like symptoms (Armstrong et al., 2020). Finally, furnished colony cages are considered to provide an improved level of welfare for hens where foot health is typically improved (Lay et al., 2011; Fiorilla et al., 2024). Hens housed in furnished cages are also considered to have better skeletal integrity, lower mortality and improved air quality (Lay et al., 2011). Although the concerns within the furnished cage setting includes the negative effects associated with perches which are also offered in other housing settings like the multi-tier. Perches in the furnished cages have been linked with increased cloacal cannibalism, potentially due to hens laying eggs on the perch leaving the cloaca exposed to other hens (Moinard et al., 1998).

To assess if housing system is a stressful environment for hens, a recent study which this chapter builds upon findings by Armstrong et al 2022 which took birds in good and poor physical condition from two housing systems, multi-tier with outdoor access and furnished colony, cages to investigate if either was linked to chronic stress by measuring DCX$^+$ density in the HF. It was found that independent of housing system, birds in poor physical condition had lower DCX$^+$ density across the HF and increased interleukin (IL)-6 gene expression in the spleen. This calls into question the role inflammation might have to play in mediating the effects of chronic stress on the reduction of DCX$^+$ density in the HF and does inflammation extend to the HF.

This chapter will explore the presence of inflammatory and non-inflammatory cytokines in the HF and spleen of those same birds where Armstrong et al 2022 found DCX$^+$ cells were reduced. This is part of is a 2x2 study building upon those findings from Armstrong et al 2022, which includes birds of good and poor physical condition from two housing systems: the free-range multi-tier (with outdoor access) and enriched colony housing systems which come with their own behavioural differences and welfare concerns. The criteria of poor physical condition for this study included a paler comb and reduced feather coverage which could come from feather pecking or friction with surfaces, reduced DCX$^+$ neuron density in these birds strengthens the welfare concerns this setting give rise to. A confined space, reduced ability to exhibit certain behaviours and potential food and water restriction which come with the conventional housing setting for hens are all potential stressors. But that does not mean that the free-range multi-tier system that this chapter will also investigate does not come without its own welfare issues.

In the rostral and caudal HF, I measured gene expression of cytokines IL-1β, IL-6, tumour necrosis factor (TNF)-α and anti-inflammatory cytokine IL-10 to determine any local inflammation. The cytokines chosen for this study are commonly involved with brain resident immune cell microglia, which either become activated in response to these cytokines or produce them locally (Walker & Spencer, 2018). The cytokines which have been selected for this study all play differing roles during inflammation, for example IL-1β is known to promote cell proliferation and mediate the immune response, whereas IL-6 secretion can be induced by IL-1β and support microglia activation (Frank et al., 2020). On the other hand, TNF-a is secreted by macrophages, including microglia during tissue injury which support microglia activation. In addition, this study measured an activation marker for microglia known as colony stimulating factor receptor-1 (CSFR-1), CSFR-1 which is present on the cell surface is bound to

CSF-1, supports the proliferation of microglia. The final gene this study measures is brain derived neurotrophic factor (BDNF) which is involved with neural plasticity including neurogenesis (Miranda et al., 2019). To determine the presence of a systemic immune response and any cytokines that could be produced following HPA axis activation, gene expression of cytokines IL-6, IL-1β, and IL-10 will also be measured in the spleen.

If inflammation is a driver of stress-led AHN reduction, this chapter hypothesises increased gene expression of the inflammatory cytokines measured and CSFR-1 in the birds of poor physical condition, in which neurogenesis was reduced. This chapter will also utilise the density of DCX^{+} multipolar and bipolar neurons provided by Armstrong et al 2022 for each individual bird and look for any direct correlations between density and gene expression. This chapter hypothesises that inflammation is a driver of stress led DCX^{+} neuron cell reduction. Therefore, there will be increased gene expression of inflammatory cytokines in the birds of poor physical condition which negatively correlates with DCX^{+} density in the HF.

2.3 Methods

2.3.1 Ethical Statement

This experiment was granted ethical approval by the Animal Welfare and Ethical Review Body at Newcastle University, project ID AWERB 549. Birds were housed and managed according to RSPCA-assured standards and DEFRA guidelines on farm, and the Home Office Code of Practice while at the University. Prior to the analysis for this study, a Home Office Schedule 1 method of euthanasia was used.

2.3.2 Animals and housing

The birds used in this study were obtained as a previous study by our lab and an in depth summary can be found here (Armstrong et al., 2022). From one day old, H&N and Hy-Line Brown pullets *(Gallus gallus domesticus)* were reared in a flat system before moving to two floor-based systems with litter at a commercial farm in the Midlands, UK. At this point the Hy-Line birds were introduced to the colony enriched cage system at 17 weeks and the H&N Pullets were introduced to the free-range multi-tier system at 16 weeks of age. Birds from both housing systems received the same course of vaccinations and at 39 weeks of age Colony housed birds received a course of the antibiotic denegard. Denegard which usually treats chronic and respiratory disease was given for poor laying performance.

When birds had lived in their respective housing for almost 1 year, a total of 12 birds each in good or poor physical body condition from the colony cage and multi-tier free range housing systems were selected by the farm's production manager. The key indicators for physical condition were feather coverage, redness of the comb and face, and body weight. This left a total of 48 birds. From the cage system, no two birds were sampled from the same cage, but birds of good and poor condition were always selected from different cages on the same tier. To ensure a sample representative of the whole colony system, birds were selected from the top, middle and bottom tiers in both an inside and outside bank. Selected birds from the multi-tier system were selected directly from the range if considered in good condition and poor condition birds were selected from the top inside tier. This ensured that the chosen birds of good condition had the ability to venture outside some of the time, where the poor condition may not have chosen to range. The birds involved with this study received all routine vaccinations, although the birds housed in the colony system received the antibiotic Denegard in addition.

2.3.3 Tissue and Processing

All spleen and HF samples used for this study were obtained from Elena Armstrong's PhD project for molecular analysis. The hippocampal tissue was dissected to then be split between rostral and caudal, alongside the spleen sample which were all weighed and then stored in RNAlater tissue storage reagent (Sigma-Aldrich, UK) for 48 hours at 4^{o}C, then frozen at -80^{o}C.

2.3.4 Quantitative real-time PCR

After HF and spleen samples had been stored frozen at -80^{o}C from the time samples were obtained until this study, RNA was extracted from rostral and caudal HF and spleen samples. This was using 1ml TriSure reagent (Bioline, London, UK) and ZR BashingBead Lysis tubes (Zymo Research, Cambridge Biosciences, UK) in a Qiagen Tissue Lyser II (Qiagen Ltd, Crawley, UK). 200µl chloroform was added to the homogenised sample, then centrifuged for 15 minutes at 13,000 rpm at 4° C. The aqueous phase was taken forward for RNA extraction using the direct-zol RNA miniprep kit (Zymo Research, Cambridge Biosciences, UK). This included the recommended DNase treatment step according to the manufacturer's protocol. 1µg RNA was transcribed using the SensiFAST cDNA synthesis kit (Meridian Bioscience, Scientific Laboratory Supplies limited, Nottingham UK), into cDNA template for quantitative real-time PCR.

Primers were designed using the primer-BLAST tool (National Centre for Biotechnology Information). The sequences can be found below (Table 1) for lamin-B receptor (LBR), colony stimulating factor receptor-1 (CSFR-1), brain derived neurotrophic factor (BDNF), tumor necrosis factor-a (TNF-α) and interleukin (IL): 10, 1β, and 6. LBR was used as a reference gene, as gene expression is consistent across all tissue. Anti-inflammatory gene IL-10 was measured in a further ratio with pro-inflammatory gene expression, to quantify the ratio of pro-inflammatory to anti-inflammatory cytokine expression for genes IL-1β, IL-6 and TNF-α. To normalise gene expression, the value for the target gene was divided by the value for LBR of the same sample to produce a ratio, which was repeated with IL-10 in place of LBR. I quantified all genes in the hippocampus and only LBR, IL-1β, IL-6 and IL-10 in the spleen. RNA from spleen tissue was limited in this study, which meant measurement of TNF-α gene expression was not possible.

Table 1: Gene-specific primers for quantitative real-time PCR

Gene-specific primers used for quantitative real-time PCR in rostral and caudal hippocampus tissue and spleen of genes colony stimulating factor-1 (CSFR-1), interleukin (IL)-10, -6, -1ß, lamin-B receptor (LBR), brain derived neurotrophic factor (BDNF) and tumor necrosis factor (TNF)-α

Gene	Orientation	Primer Sequence (5'-3')	Product Length (bp)	Accession number
CSFR-1	Forward Reverse	TCAGTCCCATTCTCACCACC CCGCACCTGGTACTTAGGTTT	104	NM_001321517.2
IL-10	Forward Reverse	GCTGAGGGTGAAGTTTGAGGA CTCTGACACAGACTGGCAGC	130	NM_001004414.4
IL-6	Forward Reverse	TCGCCTTTCAGACCTACCTG CAGATTGGCGAGGAGGGATTT	179	NM_204628.2
IL-1β	Forward Reverse	TGCCTGCAGAAGAAGCCTCG CTCCGCAGCAGTTTGGTCAT	137	XM_046931582.1
LBR	Forward Reverse	GGTGTGGGTTCCATTTGTCTACA CTGCAACCGGCCAAGAAA	80	NM_001396139.1
BDNF	Forward Reverse	GGCGGACACTTTTGAACACG TGTTTTCCTCACTGGGCTGG	74	NM_001031616.2
TNF-α	Forward Reverse	CGCTCAGAACGACGTCAA GTCGTCCACACCAACGAG	98	MF801626.1

Standards were produced by gel purification of PCR products for each gene using the MinElute gel extraction kit (Qiagen Ltd, Crawley, UK). The concentration of these standards was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) and a serial dilution was performed. Serial dilutions for each standard were used as a standard curve, for absolute quantification. Real-time PCR was run using the MIC qPCR cycler (Biomolecular Systems, UK) for 5 minutes at 95°C, then 40 cycles of 95°C for 5 seconds, 60°C for 20 seconds and 72°C for 10 seconds. Reactions were 10µl comprised of 4µl cDNA, 5µl SYBR mix (No-ROX kit, Bioline, London, UK) and 0.5µl of 10 µM of each forward and reverse primer in a 10µl final volume, producing a final primer concentration of 500 nM. For the no-template control, 4µl sterile water was added instead of cDNA. The standard curve which included 8 samples of RNA of a known concentration and samples were all ran in singlicate due to sample availability. A melting curve analysis was produced to determine the specificity of the reaction.

2.3.5 DCX$^+$ Neuron Densities

Doublecortin immunohistochemistry cell count data was provided by Elena Armstrong (Armstrong et al 2022), for rostral and caudal hippocampus samples obtained from the same brains (other hemisphere) I used for PCR analysis. Cell counts which were performed by Elena Armstrong were performed by calculating the density of DCX$^+$ cells using an optical microscope (Leica DM6B-Z, Germany), were available for multipolar and bipolar neurons and a Z score (Zi) was performed to correct for differences between staining batch.

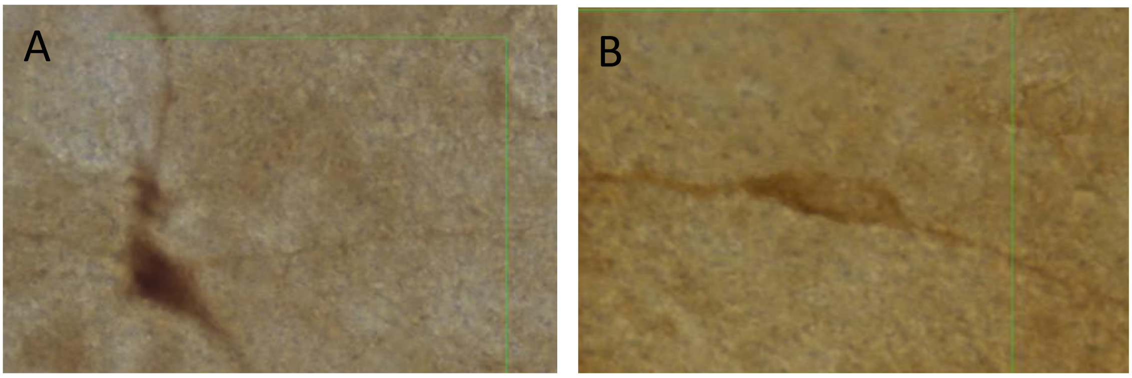

Figure 4: Doublecortin (DCX$^+$) stained Multipolar (A) and Bipolar (B) neurons in the HF at 100x magnification.

2.3.6 Statistical Analysis

This chapter uses LBR as a reference gene where all target genes were compared in a ratio of target gene to reference gene, to normalise gene expression as used in other studies (Dunn et al., 2013). For inflammatory cytokines IL-1β, TNF-α and IL-6, this was repeated with anti-inflammatory cytokine IL-10 in place of LBR to measure the expression of pro-inflammatory to anti-inflammatory cytokine expression. The natural log (LN) of each ratio was used against the reference gene LBR to calculate a Z score (Zi), to account for potential variation between RT-PCR runs which were 2 per gene due to machine capacity. The Zi was calculated by the following formula (z- μ)/σ) where z was the LN for the ratio of each sample's gene expression, μ is the mean of all samples in that run and σ was the standard deviation of all samples in the same run. For measuring DCX$^+$ neuron density, a Zi was again calculated to account for variation between staining batch. The spleen mass of each bird was also calculated using total body mass (kg) divided by spleen mass (g) to account for variations between body mass.

All graphs and statistical analysis are performed using R Studio (version 1.3.959). For all statistical analyses, housing and physical condition were included as fixed effects and Bird ID was a random effect. Where gene expression of our target genes in the spleen were analysed, a general linear model was performed using the Psych package with ANOVA. This was repeated with spleen mass. For analysis of rostral and caudal gene expression, the linear mixed effects model (LMER) with repeated measures and type 3 ANOVA was performed using the AFEX package. The Zi of DCX$^+$ multipolar and bipolar neuron density were both separately compared with the Zi of the target gene expression to determine any correlation, using again the LMER and type 3 ANOVA. Where a significant interaction or main effect was found ($p<0.05$) a pairwise comparison was performed using the estimated marginal means (EMMEANS) package. Statistical significances of $p<0.05$ are listed in text where appropriate, full statistical analysis can be found in appendix 1. All graphs were produced using GGPLOT2 in R Studio where the log ratio has been plotted.

2.4 Results

2.4.1 Spleen mass

There was a significant effect of housing system on spleen mass where birds housed in the colony system had larger spleen mass than those in the multi-tier system (F(1,43)= 7.64, P=0.008 (table 2). There was a further significant two-way interaction of spleen mass between housing system and body condition (F(1,43)= 6.37, P=0.016). This study hypothesised that there are differences in spleen mass between birds of good and poor physical condition, irrespective of housing system. Although pairwise analysis determined spleen mass is higher in birds from the Colony system with poor body condition, than birds from multi-tier housing in good body condition (T(43)= -2.71, P=0.04) (table 3, table 2, figure 5). Spleen mass was also significantly changed in the birds of poor condition from both housing systems where birds from the colony system in poor condition had larger spleen mass than poor condition birds from the multi-tier system (table 2).

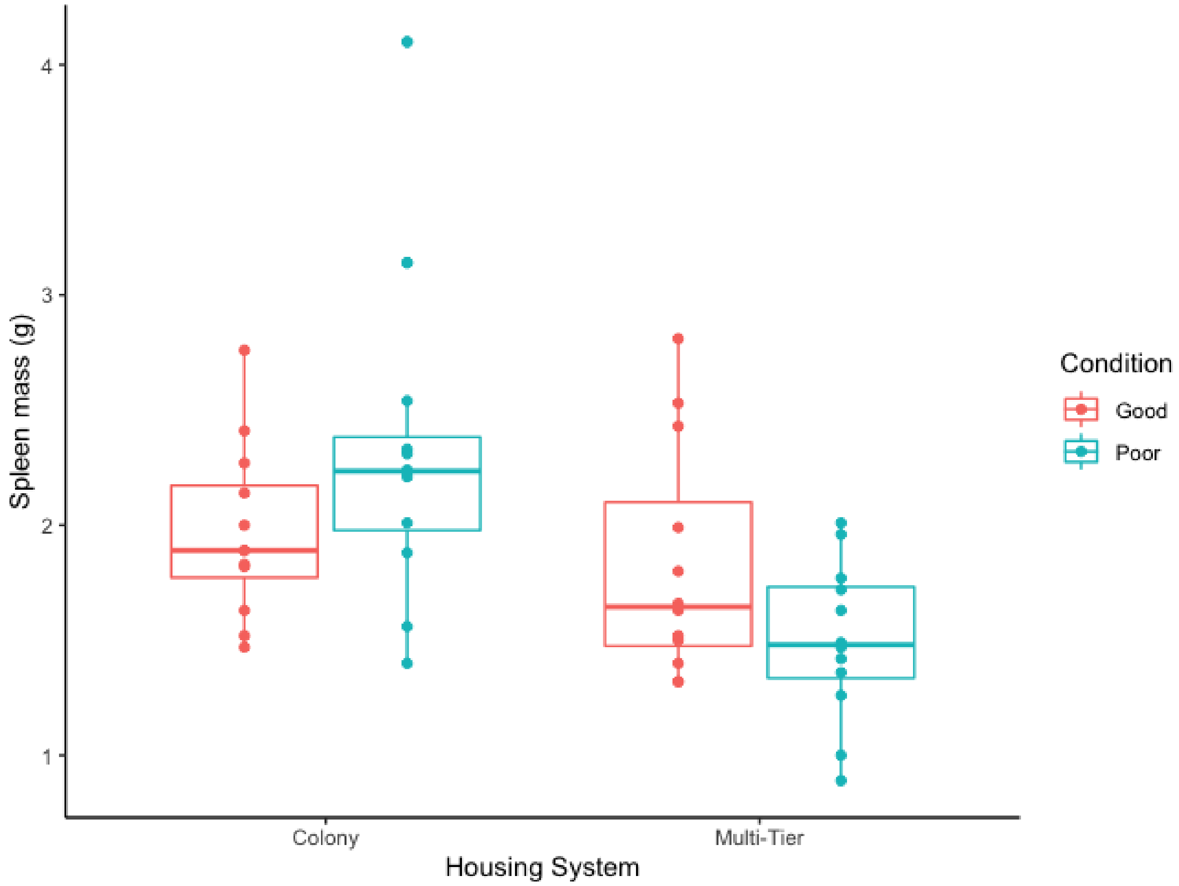

Figure 5: Spleen mass is increased in the birds of poor condition from the colony housing system.

A boxplot showing the spleen mass (g) for each bird of good and poor physical condition from colony and multi-tier housing systems. The line visualises the median of each group with the hinges representing the upper and third quartile. Sample n= 11 Colony good, 12 Colony poor, 12 Multi-tier good, 12 Multi-tier poor.

Table 2: Spleen mass in birds of good and poor condition from two housing systems

Statistical output from the linear mixed model with three-way ANOVA of spleen mass (g) between bird mass (kg) of birds in good and poor physical condition from multi-tier and colony housing systems. Significant interactions (p<0.05) are highlighted in bold.

Spleen mass	**Bird mass**	**F(1,43)= 9.0197, P=0.00439**
	System	**F(1,43)= 7.6453, P=0.008351**
	Condition	F(1,43)= 0.9988, P= 0.323189
	System: Condition	**F(1,43)= 6.3681, P=0.015396**

Table 3: Estimated marginal means of spleen mass in birds of good and poor condition from two housing systems.

Pair-wise analysis of the estimated marginal means (emmeans) of significant interactions between spleen mass, body condition and housing system. Significant interactions (p<0.05) are highlighted in bold.

Spleen mass	**Colony - Multi-Tier**	**T(43)= 2.613, P=0.0123**	**Colony increase**
	Colony - Good Multi-Tier Good	T(43)= 0.149, P=0.9988	**-**
	Colony Good – Colony Poor	T(43)= -2.452, P=0.0824	**-**
	Colony - Good Multi-Tier Poor	T(43)= 1.027) P=0.7349	**-**
	Multi-Tier Good – Colony Poor	**T(43)= -2.714, P=0.0453**	**Colony poor increase**
	Multi-Tier Good – Multi-Tier Poor	T(43)=0.966, P=0.7692	**-**
	Colony Poor – Multi-Tier Poor	**T(43)= 3.645, P=0.0039**	**Colony poor increase**

2.4.2 Gene expression in the spleen

To determine the presence of systemic inflammation, I measured cytokine gene expression in the spleen (table 4). Similarly to the HF which will be discussed later, there was a significant effect of housing system on IL-10 expression (F(1,27)=25.7846, P=0.00000784) (table 4) where expression was higher in the colony system. There was a further significant effect of housing system with IL-6 expression when compared in a ratio with IL-10 (F(1,38)=6.8092, P=0.01243) suggesting a greater pro-inflammatory to anti-inflammatory gene expression, but not when compared with housekeeping gene LBR (table 4). Pair-wise analysis shown a higher ratio of IL-6/IL-10 in the spleen of birds from the multi-tier system (figure 6A), but there was no increase in IL-6 when comparing with housekeeping gene LBR. There was no statistical difference in IL-6/LBR expression in the spleen between bird condition F(1,44)=0.0385, P=0.8454, suggesting the significance of IL-6/IL-10 expression between colony and multi-tier birds might be driven by differences in IL-10 expression. Finally, the condition or housing of the bird did not impact IL-1β/LBR and IL-1β/IL-10 expression, nor was there any differences in expression across the HF (table 4).

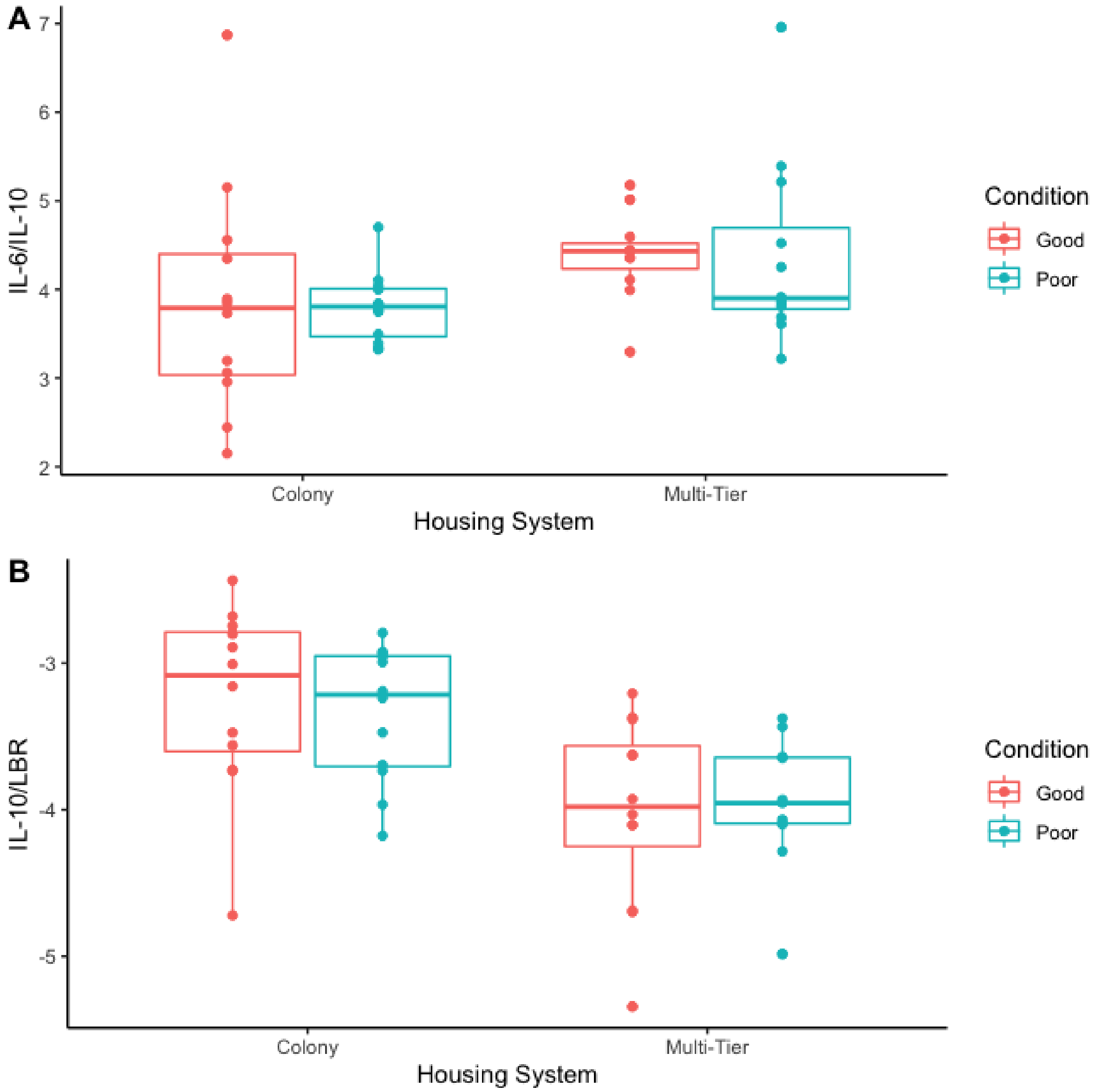

Figure 6: IL6/IL-10 gene expression is increased in Colony housed birds and IL-10/LBR gene expression is increased in multi-tier birds.

Box plot showing a ratio of the expression of IL-6 and IL-10 (A), IL-10 and LBR (B) in the spleen of birds housed in either a multi-tier or colony system, both of good or poor physical condition. The line visualises the median of each group with the hinges representing the upper and third quartile.
Plot A: Sample n= 12 Multi-tier poor, 11 Multi-tier good, 12 Colony poor, 12 Colony good.
Plot B: Sample n= 11 Multi-tier poor, 12 Multi-tier good, 12 Colony poor, 12 Colony good.

Table 4: Gene expression in the spleen of birds in good and poor condition from two housing systems

Statistical output from the linear model with thee-way ANOVA of gene expression in a ratio with LBR or IL-10 (Zi) in the spleen of birds in good and poor physical condition in birds from colony and multi-tier housing systems. Significant interactions (p<0.05) are highlighted in bold.

IL-1β/LBR	System	F(1,43)=1.6960, P=0.2006
	Condition	F(1.43)=1.1035, P=0.3001
	System: Condition	F(1,43)=0.2352, P=0.6305
IL-1β/IL-10	System	F(1,35)=0.0589, P=0.8096
	Condition	F(1,35)=1.9208, P=0.1738
	System: Condition	F(1,35)=0.5704, P=0.4548
IL-6/LBR	System	F(1,44)=0.0140, P=0.9064
	Condition	F(1,44)=0.0385, P=0.8454
	System: Condition	F(1,44)=0.0273, P=0.8696
IL-6/IL-10	**System**	**F(1,38)=6.8092, P=0.01243**
	Condition	F(1,38)=0.0023, P=0.96192
	System: Condition	F(1,38)=0.0187, P=0.89194
IL-10/LBR	**System**	**F(1,27)=25.7846, P=0.00000784**
	Condition	F(1,27)=0.0027, P=0.9588
	System: Condition	F(1,27)=0.5114, P=0.4784

2.4.3 Gene Expression in the hippocampus

Of all genes measured in the HF, there was a significant two-way interaction between the HF subdivision and housing system for IL-10/LBR gene expression (F(1,35)=7.9162, P=0.007926) (table 5). Pair-wise analysis shows that in the caudal HF only, IL-10 expression is higher in birds housed in the Colony system, compared to the multi-tier (t(66)=2.656, P=0.0476), with no effect of condition (figure 7). However, expression is higher overall in the rostral vs caudal HF (figure 7).

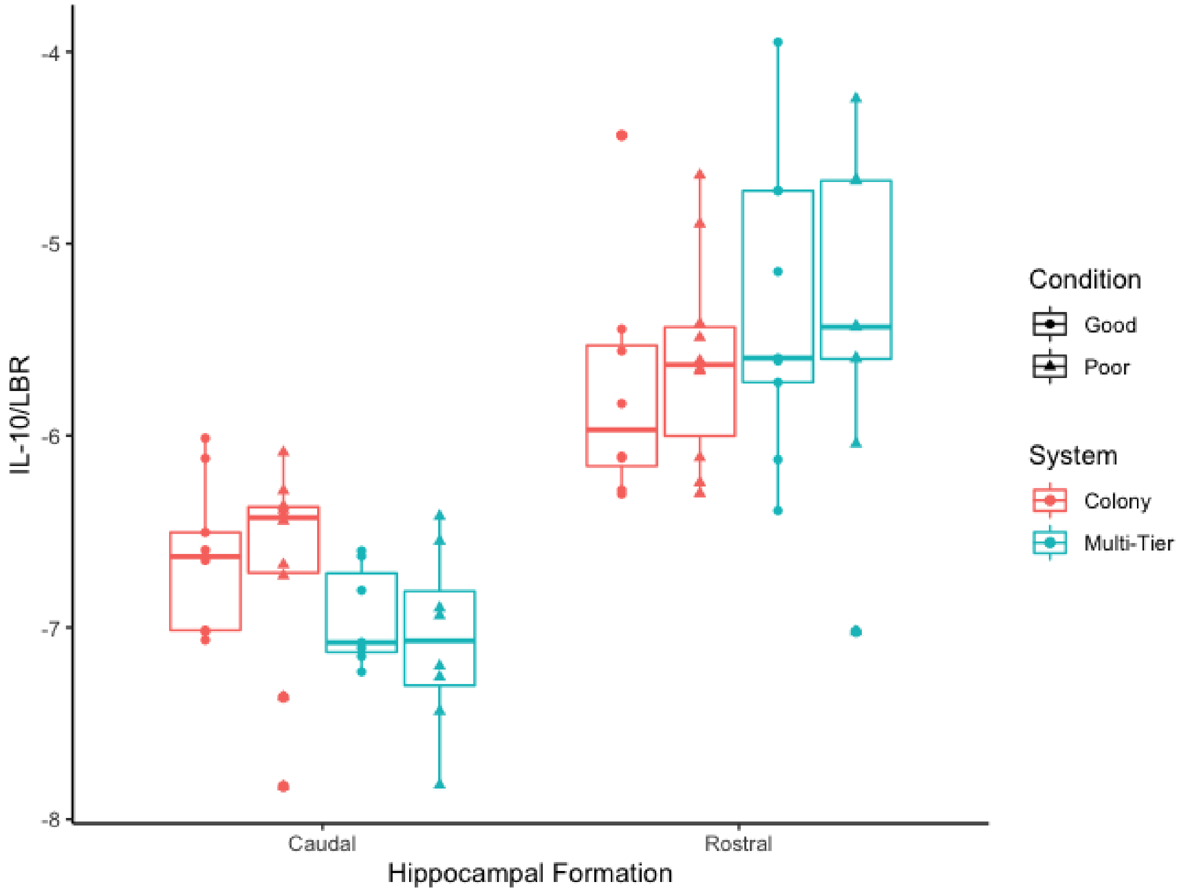

Figure 7: IL-10/LBR gene expression is increased in the caudal compared to rostral HF of birds housed in the colony system, IL-10/LBR is higher in the rostral vs caudal HF of all groups.

Boxplot showing a ratio log of the expression of IL-10/LBR in the rostral and caudal hippocampus of birds housed in either a multi-tier or colony system, both of good or poor physical condition. The line visualises the median of each group with the hinges representing the upper and third quartile. Sample n= Multi-tier poor: 9 rostral 8 caudal, Multi-tier good: 9 rostral 7 caudal, Colony poor: 10 rostral 10 caudal, Colony good: 8 rostral 9 caudal.

All further cytokines measured were statistically non-significant between housing system or physical condition and can be observed in table 5 indicating no change in gene expression, nor was there a difference in expression between the HF subdivisions. Furthermore, there were no significant differences amongst expression of microglia activation marker CSFR-1 or BDNF between condition, the HF subdivision or housing system.

2.4.4 DCX$^+$ Neuron density and gene expression

Armstrong et al 2022 found lower density of DCX$^+$ multipolar neurons across the whole HF of birds in poor condition than good and a trend towards lower bipolar density, but no effect of housing systems (Armstrong et al., 2022). Therefore, I ran a correlation analysis between gene expression in the rostral and caudal HF and multipolar and bipolar density. This analysis found there is no direct correlation between any of the genes measured and DCX$^+$ density of neither multipolar (table 6) or bipolar density (table 7). There was however an almost significant effect of IL-6/LBR expression on multipolar density (F(1,60)=3.7868, P=0.05632) (table 6), where expression of this gene did not show statistical significance ($p<0.05$), expression appears to be secreted at larger quantities in the caudal HF when density is greater and the almost reverse in the rostral HF (figure 8).

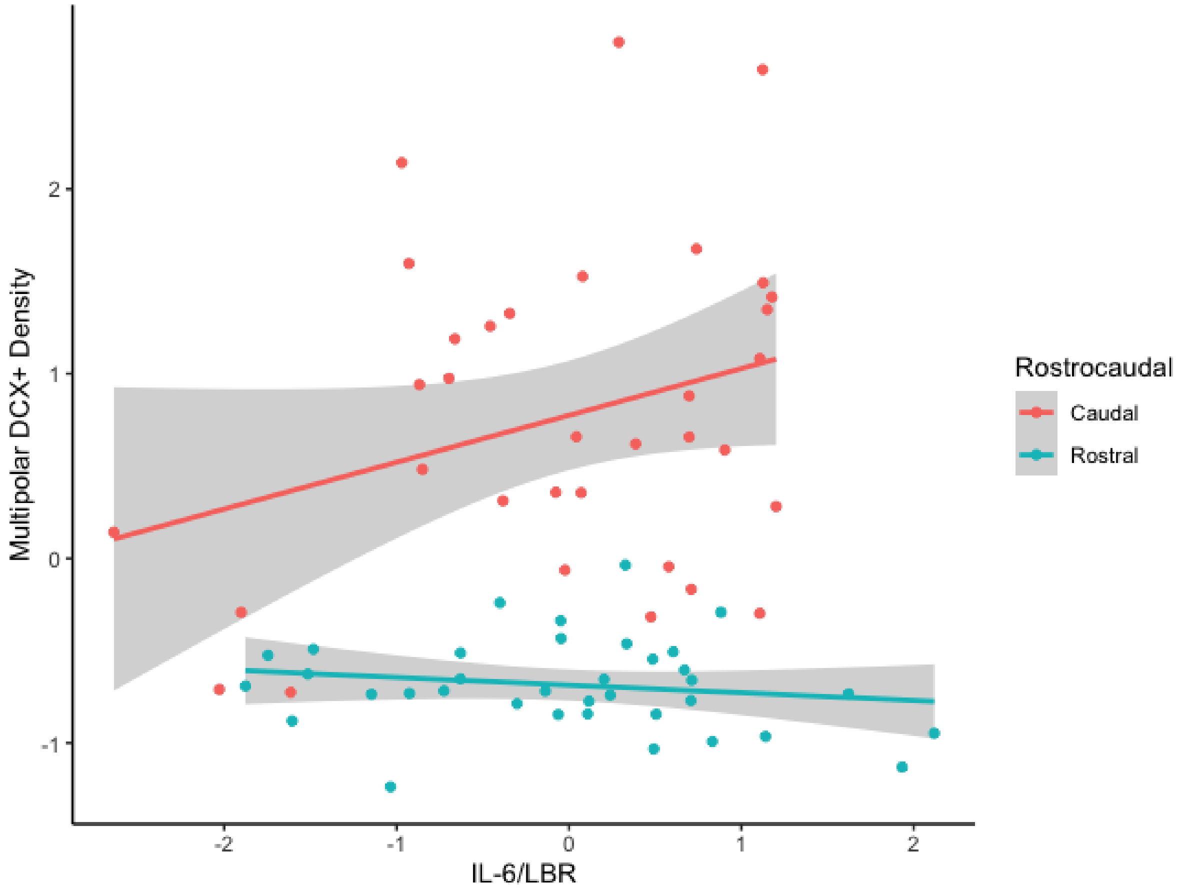

Figure 8: Correlation between Multipolar DCX$^+$ Density in the rostral and caudal HF and IL-6/LBR gene expression

The correlation between Multipolar DCX$^+$ density and a ratio log of IL-6/ LBR gene expression in the rostral and caudal hippocampus. Shading represents the standard error of the slope. Sample n= 36 rostral, 34 Caudal.

2.5 Discussion

This chapter hypothesised that where Armstrong et al 2022 found DCX$^+$ density was decreased in the HF of birds in poor physical condition, there would be an increase in inflammatory cytokines in this study. To explore the possibility of the immune system mediating the reduction of DCX$^+$ in birds of poor physical condition as a first step I measured gene expression of inflammatory cytokines in the hippocampus which may have been produced by cells in the HF or crossing the blood brain barrier (BBB). Initial results indicate bird condition had no impact on the expression of any genes measured across the hippocampus, but housing system did. The ratio of IL-10/LBR was significantly higher in the caudal HF of birds housed in the colony system. There were no signs of local inflammation within the HF, nor were there any changes in BDNF and CSFR-1 expression amongst groups. Together these findings indicate the hypothesis is not supported. Increased gene expression of the cytokines measured in this study on this occasion are not the driver of stress-led reduction of DCX^+ neurons in birds of poor condition based on this data. However, the samples used for this analysis were all obtained within the same timeframe and may not consider changes to cytokine expression throughout the period the bird is considered stressed. For example, it may be possible that there is an increase of inflammatory cytokines during the initial period of chronic stress where the bird might have first been identified to have poor physical condition which future study should consider. As Armstrong et al 2022 found no changes in DCX^+ density within housing systems and only within body condition, the changes found in cytokine expression between housing systems opposed to physical condition suggest inflammatory cytokines are not driving DCX^+ reduction in the HF at the time these samples were obtained.

As a next step inflammatory cytokines IL-1β, IL-6 and anti-inflammatory IL-10 were measured in the spleen, alongside spleen mass itself. Similarly to the HF, IL-10 expression differs amongst housing only where expression is significantly higher in the colony system (figure 6). In addition, the ratio of IL-6/IL-10 expression in the spleen was significantly increased in birds housed in the multi-tier system which could be an indicator of inflammation. This may indicate the presence of a systemic immune response with the increased ratio of IL-6/IL-10, although because IL-6/LBR was not significantly different amongst any group this remains uncertain. However, when IL-6/LBR spleen expression was measured in these same samples previously there was an increase in expression in the birds of poor condition (Armstrong et al., 2022). Given the samples measured in the present study are the same as (Armstrong et al., 2022) this was an unexpected result but could be due to the time period between both studies including cDNA storage and the thawing and refreezing process. As previously mentioned, the present study measured gene expression in singlicate, it is unclear which approach Armstrong et al 2022 chose. However, pair-wise analysis did determine the results in the present study did point towards the same trend as (Armstrong et al., 2022) where IL-6/LBR was higher in the birds of poor condition overall, although not significant. This does point towards potential inflammation and IL-6, which should be explored in future studies of chronic stress with consideration to multiple time points during chronic stress.

To explore this further I ran a correlation analysis directly on multipolar and bipolar DCX$^+$ density and gene expression on rostral and caudal HF, irrespective of bird group. Although no significant correlation between multipolar and bipolar density was present, there was an almost significant correlation between multipolar density and IL-6/LBR expression which differed between the HF subdivisions (table 6, table 7). As shown in figure 5 in the caudal HF IL-6/LBR expression increases as multipolar density increases, following a positive trend which was not present in the rostral HF which stayed consistent. On this occasion, no other genes measured pointed towards any relationship with DCX$^+$ neuron density.

Inflammatory cytokines including IL-6, which was increased in the spleen of birds from the multi-tier system and had an upward trend in the birds of poor condition, are typically secreted to initiate the neuroimmune response, leading to the activation of microglia and further inflammatory secretion in the brain (de Miranda *et al*., 2017; Guadagno *et al*., 2015). As there were no differences in IL-6/LBR expression in the HF and expression increased as multipolar density increased in the caudal HF, it appears that the effect of IL-6 may be driven by the systemic response rather than a locally produced response by microglia which could occur at a later stage. For example, a study in broiler chickens found IL-6 expression to be increased in the spleen following a period of heat stress (Al-Zghoul et al., 2019). The chronic elevation of IL-6 is additionally associated with poor welfare and inflammation in mammalian research. A study which used IL-6^{KO} mice subject to repeated social defeat discovered an absence of anxiety and social avoidance which was present in the IL-6$^+$ mice. However, this same study found increased microglia with an ameboid phenotype in the stress group where cell numbers did not differ amongst IL-6^{KO} or IL-6$^+$ mice. Therefore this data may suggest increased IL-6 expression in the spleen is a consequence of chronic stress which occurs alongside reduced AHN in our birds. But alongside the results of this study we cannot draw any conclusions and can only speculate that IL-6 expression is related to reduction of AHN and may not the whole cause.

The immune system's response to chronic stress is a cascade of inflammatory cytokines and cell types including microglia. Following peripheral inflammatory cytokine secretion, microglia are activated leading to further cytokine secretion in the hippocampus. The IL-6 increase observed in the spleen of birds from the multi-tier alongside IL-10 increase in the hippocampus of birds from the colony system of this chapter, could be due to this orchestrated inflammatory response. Although as there was no correlation between DCX^+ density and the expression of these genes and no reduction in DCX^+ density was found between housing systems (Armstrong et al., 2022), this may not be stress driven. It is likely there is some involvement with microglia, however CSFR-1 which is expressed by activated microglia was not expressed differently between housing systems or condition. Although this might be a somewhat unexpected result, it does point towards limitations within this study. As previously mentioned, microglia are active with different functions depending on the local environment (Reemst et al., 2016) with CSFR-1 being expressed in all states (Colonna & Butovsky, 2017).

Despite some emerging evidence for a positive role of microglia within the avian species, little work has been done to investigate the inflammatory role of microglia potentially leading to DCX^+ neuron reduction as it has in mammals. One study in Japanese Quail measured CSFR-1 in the hippocampus and other regions, alongside IL-1β and IL-10 (Walker et al., 2019b). This study targeted pre and post-natal stress where Quail subject to post-natal stress disrupted IL-1β/IL-10 expression in the hippocampus where IL-10 was increased in comparison to IL-1β in all Quail, like the birds in the colony housing system in this study. This study also measured CSFR-1 expression which was only reduced in the hypothalamus and pituitary gland of birds subject to pre-natal stress (Walker et al., 2019b). It is however worth noting that the study did not investigate neurogenesis or hippocampal volume as the previously mentioned studies did (Diniz et al., 2016; Viana et al., 2013) therefore it is unclear if these changes result in any hippocampal changes. In the mammalian literature microglia have been linked with producing excess inflammation and driving neuron loss through phagocytosis, which along with all previously mentioned studies demonstrating the potential positive role of microglia, could explain why there were no differences in CSFR-1 expression for the present study. In response to tissue damage, microglia can produce large quantities of nitric oxide (NO) through iNOS expression.

This concept has been demonstrated in the Avian species through LPS administration and upregulation of inflammatory cytokines. (Sierra et al., 2014) used LPS to treat microglia in the Quail retina. LPS led to a transition from ramified microglia which are considered to have beneficial effects on neurons, to the ameboid type which upregulate iNOS and secrete NO. Although not demonstrated, Sierra et al 2014 speculate NO production by upregulated iNOS in quail might lead to neuronal death by peroxynitrate which is a by-product from oxidation of NO with superoxide. Furthermore, it has been shown that in the mammalian hippocampus naturally occurring neuronal death is mediated by superoxide production (Marín-Teva et al., 2004). The transition between microglia having positive and negative effects on neurogenesis occur all while they remain active, indicating the measurement of CSFR-1 in the present study might not be the most useful tool to determine if microglia are acting inflammatory leading to the reduction in neurons of our poor condition birds. In future chapters, it would be useful to determine the morphology or phenotype of microglia between ramified and ameboid to determine if there’s any further sign of inflammation alongside measurement of iNOS. This could also explain why none of the inflammatory cytokines measured were upregulated in the poor condition birds, as ameboid microglia often come as a response to inflammatory cytokines (Sierra et al., 2014).

The main findings from this chapter were birds housed in the colony system had increased IL-10 gene expression in the caudal HF and spleen. As described by Armstrong et al., 2022) all birds received identical vaccination programmes, but the colony housed birds received antibiotic treatment due to poor laying performance. These birds received a course of Denegard at 39 weeks of age, which could explain the increase of IL-10. Denegard contains tiamulin which is a semi-synthetic antibiotic which inhibits bacterial protein synthesis. It is unknown if this antibiotic leads to increased IL-10, although treatment of colistin in pigs which is another antibiotic has lead to increased serum, duodenum and jejunum IL-10 levels (Koutsoumanis et al., 2021). Although this is not certain, this could explain the increase of IL-10 in the spleen and caudal HF of birds from the colony housing system.

Still, measuring gene expression through mRNA transcripts means we are unable to account for the varying level of transcription in individual cells, as RNA concentration can vary amongst individual cells ultimately impacting global expression levels (Padovan-Merhar *et al*., 2015). Instead, and similar to what was done for neurogenesis by DCX^+ staining, cell density can be measured as an indicator of changes in cellular processes (Grover *et al.*, 2011) which would otherwise be undetectable by mass measurement through qPCR.

A final point of consideration from this study were the draw backs from selecting birds from their long-term environments rather than a protocol like UCMS which is commonly used to effectively cause chronic stress and has been demonstrated to lead to AHN reduction (Gualtieri et al., 2019). Due to the birds used in the present study being selected from a farm having the opportunity to live amongst other birds for long periods of time, it was unclear how long these birds may have been subject to poorer welfare which could have led to the upregulation of inflammatory cytokines which could have led to reduced AHN at any given point. As demonstrated by (Liu et al., 2015) TNF-α is increased in mice 4 weeks into a UCMS protocol and IL-1β is increased in patients with depression and detrimental to neurogenesis (Zunszain et al., 2012). Although these cytokines were not increased within any specific group within the present study, nor did they form any relationship with multipolar or bipolar density, it could explain why IL-10 is increased in the caudal HF and spleen of birds within the colony system to restore homeostasis following any potential increases of inflammatory cytokines. Furthermore, this study used H&H and Hy-line brown pullets, whereas a study by Wei et al 2019 used Lohman White where they found the presence of a KBF which is considered a stressor (Armstrong et al 2020), lead to increased inflammatory cytokine expression in the brain. Although there are no direct comparisons between bird strain in relation to chronic stressors and cytokine expression, they can show differences in response to fear. For example, one study found Rhode Island red spent more time in TI than the White Leghorn strain (Nelson et al., 2020). However, there are also no known differences in hippocampal plasticity during chronic stress across the strains. In future studies it is important to consider these strain differences throughout chronic stress and explore how this impacts inflammatory cytokines throughout. Some immune parameters including lymphocytes are considered to be consistent across strain (Schmucker et al., 2021). However, this is considered to be as the bird generally ages rather than in response to stress.

2.6 Concluding remarks

In conclusion, the role of the immune system as a driver of reduced AHN in hens with chronic stress remains unclear. This chapter demonstrates a potential discrepancy between housing systems and the effect on inflammatory cytokines, but not body condition as originally expected. The condition of the bird had no relationship with any of the genes we measured, nor did those cytokines have any relationship with DCX^+ neuron density. What was found, was the possibility of the early stages of the immune response to stress through increased IL-10 gene expression in the spleen of birds from the colony system. However, given that these birds had increased IL-10 in the spleen and caudal HF where DCX density of both neurons was no different between housing systems, it is difficult to determine if this comes as a result of the first steps of the immune response or the restoration of homeostasis following an episode of inflammation or as a result of antibiotic treatment. To investigate further, it would be beneficial to explore further markers of the inflammatory response and activation states of microglia, such as the morphology and secreted products like iNOS as signs of inflammation and phagocytosis.

2.7 Appendix 1

Table 5: Gene expression in the rostral and caudal HF of birds in good and poor condition from two housing systems

Statistical output from the linear mixed model with three-way ANOVA of gene expression in a ratio with LBR or IL-10 (Zi) in the rostral and caudal HF of birds in good and poor physical condition from colony and multi-tier housing systems. Significant interactions (p<0.05) are highlighted in bold.

IL-1β/LBR	System	F(1,33)= 0.1291, P=0.7216
	Condition	F(1,33)=0.3590, P=0.5531
	Rostrocaudal	F(1,29)= 0.0031, P= 0.9560
	System: Condition	F(1,33)= 0.1318, P= 0.7189
	System: Rostrocaudal	F(1,29)= 0.0788, P= 0.7809
	Condition: Rostrocaudal	F(1,29)= 0.0397, P= 0.8434
	System: Condition: Rostrocaudal	F(1,29)= 0.1564, P= 0.6954
IL-1β/IL-10	System	F(1,34)=0.0929, P= 0.7624
	Condition	F(1,34)= 0.2789, P= 0.6008
	Rostrocaudal	F(1,26)= 0.0011, P= 0.9741
	System: Condition	F(1,34)= 0.2587, P= 0.6142
	System: Rostrocaudal	F(1,26)= 0.7609, P= 0.3909
	Condition: Rostrocaudal	F(1,26)= 0.0018, P= 0.9666
	System: Condition: Rostrocaudal	F(1,26)= 0.2627, P= 0.6125
IL-6/LBR	System	F(1,36)= 3.4236, P= 0.07248
	Condition	F(1,36)= 1.6624, P= 0.20549
	Rostrocaudal	F(1,36)= 0.0032, P= 0.95510
	System: Condition	F(1,36)= 0.1281, P= 0.72248
	System: Rostrocaudal	F(1,36)= 0.0419, P= 0.83890
	Condition: Rostrocaudal	F(1,36)= 0.2786, P= 0.60085
	System: Condition: Rostrocaudal	F(1,36)= 0.5293, P= 0.47159
IL-6/IL-10	System	F(1,36)= 2.6063, P= 0.1151
	Condition	F(1,36)= 0.0160, P= 0.9001
	Rostrocaudal	F(1,35)= 0.0108, P= 0.9176
	System: Condition	F(1,36)= 0.8722, P= 0.3565
	System: Rostrocaudal	F(1,35)= 1.1043, P= 0.3004
	Condition: Rostrocaudal	F(1,35)= 1.0639, P= 0.3093
	System: Condition: Rostrocaudal	F(1,35)= 0.0619, P= 0.8050

TNF-α/LBR	System	F(1,37)= 0.0232, P= 0.8799
	Condition	F(1,37)= 0.2600, P= 0.6131
	Rostrocaudal	F(1,33)= 0.0782, P= 0.7814
	System: Condition	F(1,37)= 0.4616, P= 0.5010
	System: Rostrocaudal	F(1,33)= 0.0633, P= 0.8028
	Condition: Rostrocaudal	F(1,33)= 0.8632, P= 0.3594
	System: Condition: Rostrocaudal	F(1,33)= 2.3531, P= 0.1343
TNF-α/IL-10	System	F(1,35)=0.3238, P=0.57293
	Condition	F(1,35)=0.4235, P=0.51939
	Rostrocaudal	F(1,29)=0.0419, P=0.83924
	System: Condition	F(1,35)=0.8066, P=0.37517
	System: Rostrocaudal	F(1,29)=3.3983, P=0.07533
	Condition: Rostrocaudal	F(1,29)=1.3842, P=0.24880
	System: Condition: Rostrocaudal	F(1,29)=2.2676, P=0.14274
IL-10/LBR	System	F(1,36)= 0.8333, P= 0.36731
	Condition	F(1,36)= 0.0000, P= 0.99548
	Rostrocaudal	F(1,35)= 0.0406, P= 0.84152
	System: Condition	F(1,36)= 0.2271, P= 0.63652
	System: Rostrocaudal	**F(1,35)=7.9162, P= 0.007926**
	Condition: Rostrocaudal	F(1,35)= 0.0457, P= 0.83191
	System: Condition: Rostrocaudal	F(1,35)= 0.0396, P= 0.84340
CSFR-1/LBR	System	F(1,36)= 2.3964, P= 0.1303
	Condition	F(1,36)= 0.0691, P= 0.7942
	Rostrocaudal	F(1,36)= 0.0278, P= 0.8686
	System: Condition	F(1,36)= 0.0458, P= 0.8317
	System: Rostrocaudal	F(1,36)= 0.0005, P= 0.9820
	Condition: Rostrocaudal	F(1,36)= 0.8326, P= 0.3676
	System: Condition: Rostrocaudal	F(1,36)= 0.3170, P= 0.5769
BDNF/LBR	System	F(1,36)= 3.5349, P= 0.06819
	Condition	F(1,36)= 0.5168, P= 0.47683
	Rostrocaudal	F(1,36)= 0.0121, P= 0.91295
	System: Condition	F(1,36)= 0.1602, P= 0.69134
	System: Rostrocaudal	F(1,36)= 0.1474, P= 0.70332

	Condition: Rostrocaudal	F(1,36)= 0.6790, P= 0.41533
	System: Condition: Rostrocaudal	F(1,36)= 0.2957, P= 0.58997

Table 6: Correlation between multipolar neuron density and gene expression in the rostral and caudal HF of birds in good and poor condition from two housing systems

Statistical output from the linear mixed model with three-way ANOVA of the correlation between Multipolar DCX+ density (Zi) and gene expression in a ratio with LBR and IL-10 (Zi) in the rostral and caudal HF of all birds

Multipolar Density (DCX^+)	IL-1β/LBR	F(1,53) =1.3247,P= 0.2549
	HF	**F(1,34)= 84.2403, P= 9.97e-11**
	IL-1β/LBR : HF	F(1,53)= 2.5043, P= 0.1195
Multipolar Density (DCX^+)	IL-1β/IL-10	F(1,48)=1.4037, P= 0.2419
	HF	**F(1,34)=84.2203 , P=9.845e-11**
	Gene expression : HF	F(1,48)=2.4915, P=0.1210
Multipolar Density (DCX^+)	IL-6/LBR	F(1,60)=1.9516, P=0.16753
	HF	**F(1,37)=96.2594, P=5.829e-12**
	IL-6/LBR : HF	F(1,60)=3.7868, P=0.05632
Multipolar Density (DCX^+)	IL-6/IL-10	F(1,65)= 2.7169,P=0.1040
	l HF	**F(1,37)=93.7337, P= 9.633e-12**
	IL-6/IL-10 : HF	F(1,65)=0.8668, P=0.3552
Multipolar Density (DCX^+)	TNF-α/LBR	F(1,65)=0.0037, P=0.9514
	Rostral and Caudal HF	**F(1,38)=89.2116,P=1.621e-11**
	TNF-α/LBR : HF	F(1,65)=1.1131, P=0.2953
Multipolar Density (DCX^+)	TNF-α/IL-10	F(1,63)=0.0014, P=0.9705
	HF	**F(1,38)=88.2313, P= 1.875e-11**
	TNF-α/IL-10 : HF	F(1,63)=0.4448, P=0.5072

Multipolar Density (DCX$^+$)	IL-10/LBR	F(1,65)=0.0002, P=0.9886
	HF	**F(1,38)=87.6148,P=2.063e-11**
	IL-10/LBR : HF	F(1,65)=0.0257, P=0.8731
Multipolar Density (DCX$^+$)	BDNF/LBR	F(1,61)=1.2574, P=0.2665
	HF	**F(1,37)=90.5999, P= 1.388e-11**
	BDNF/LBR : HF	F(1,61)=0.9448, P=0.3348
Multipolar Density (DCX$^+$)	CSFR-1/LBR	F(1,58)=0.2778, P=0.6001
	HF	**F(1,37)=88.6169, P= 1.831e-11**
	CSFR-1/LBR : HF	F(1,58)=0.4812, P=0.4906

Table 7: Correlation between bipolar neuron density and gene expression in the rostral and caudal HF of birds in good and poor condition from two housing systems

Statistical output from the linear mixed model with three-way ANOVA of the correlation between Bipolar DCX^{+} density (Zi) and gene expression in a ratio with LBR and IL-10 (Zi) in the rostral and caudal HF of all birds

Bipolar Density (DCX^{+})	IL-1β/LBR	F(1,53)=1.0698, P= 0.3057
	HF	**F(1,34)=82.4671, P=1.293e-10**
	IL-1β/LBR : HF	F(1,53)=2.4099, P=0.1265
Multipolar Density (DCX^{+})	IL-1β/IL-10	F(1,48)=0.8772, P=0.3536
	HF	**F(1,34)=81.0933, P=1.562e-10**
	Gene expression : HF	F(1,48)=1.6855, P=0.2003
Bipolar Density (DCX^{+})	IL-6/LBR	F(1,60)=0.3590, P=0.5513
	HF	**F(1,37)=70.9051, P=3.245e-10**
	IL-6/LBR : HF	F(1,60)=1.2118, P=0.2754
Bipolar Density (DCX^{+})	IL-6/IL-10	F(1,65)=0.6391, P=0.4269
	HF	**F(1,37)=70.1027, P=4.152e-10**
	IL-6/IL-10 : HF	F(1,65)=0.0359, P=0.8503
Bipolar Density (DCX^{+})	TNF-α/LBR	F(1,65)=1.0859, P=0.3012
	HF	**F(1,38)=70.8633, P=3.239e-10**
	TNF-α/LBR : HF	F(1,65)=0.5955, P=0.4431
Bipolar Density (DCX^{+})	TNF-α/IL-10	F(1,63)=0.0180, P=0.8938
	HF	**F(1,38)=70.2943, P=3.574e-10**
	TNF-α/IL-10 : HF	F(1,63)=1.2221, P=0.2731
Bipolar Density (DCX^{+})	IL-10/LBR	F(1,65)=0.8553, P=0.3584
	HF	**F(1,38)=70.1012, P=3.704e-10**
	IL-10/LBR : HF	F(1,65)=0.2641, P=0.6090

Bipolar Density (DCX^{+})	BDNF/LBR	F(1,61)=0.0056, P=0.9406
	HF	**F(1,37)=68.9077, P=4.782e-10**
	BDNF/LBR : HF	F(1,61)=0.0007, P=0.9796
Bipolar Density (DCX^{+})	CSFR-1/LBR	F(1,58)=0.4325, P=0.5134
	HF	**F(1,37)=69.3809, P=4.325e-10**
	CSFR-1/LBR : HF	F(1,58)=0.0007, P=0.9787

CHAPTER 3: DO KEEL BONE FRACTURES ALTER EXPRESSION OF INFLAMMATION-RELATED GENES IN LAYING HENS?

3.1 Abstract

In a recent study keel bone fractures (KBF) have been shown to decrease hippocampal plasticity in correlation with fracture severity and lead to the onset of depressive-like symptoms in laying hens, which is worsened by fracture severity. Decreased hippocampal plasticity is considered a marker of chronic stress in hens, although it is not clear if chronic stress and reduced hippocampal plasticity involves inflammatory cytokines. To explore a potential role for inflammatory cytokines during reduced hippocampal plasticity caused by KBF, this chapter measures gene expression of inflammatory cytokines in the hippocampus. This includes interleukin (IL) IL-1β, IL-6 and anti-inflammatory IL-10 alongside other markers of inflammation in laying hens with severe and minimal KBF. Results show that there is no difference in expression of these genes in the rostral or caudal hippocampal formation (HF) between minimal or severe fractures. Expression of IL-1β increases whilst IL-10 decreases in the rostral HF of hens who acquired fractures earlier. IL-6 expression increases in hens who acquired fractures later, whilst there is no significant relationship between fracture and expression in the caudal HF, neither was there any relationship between expression and doublecortin (DCX^+) density. Expression of these genes does not predict DCX^+ density indicating no relationship between inflammation and reduced hippocampal plasticity, but this does point towards potential inflammation in the rostral HF of birds with KBF.

3.2 Introduction

Keel bone fractures (KBFs) pose a severe welfare concern for laying hens. Between 20 to 96% of birds within a commercial flock are reported to have some level of fracture across many countries including but not limited to the UK (Toscano et al., 2020), Denmark (Thøfner et al., 2021), and Canada (Petrik et al., 2014). Many KBFs can be caused from colliding with perches and other furnishings like support beams and drinkers (Weeks & Nicol, 2006), alongside recent evidence suggesting an association between KBF prevalence and larger egg weight (Thøfner et al., 2021). As a result it is estimated that the prevalence of KBF increases as the bird ages rising from 5.5% of birds within the flock at the onset of lay and up to 97% by the end of the production cycle (Sandilands et al., 2009). As the ban of battery cages in 2012 within the EU came into practice, implications for the hens' welfare arose as hens were alternatively housed in extensive systems (Sandilands et al., 2009). As birds became able to express more natural behaviour and perches provided an opportunity to increase leg and bone strength through loading exercise, this comes with a greater chance for bone fractures, including the keel bone (Weeks & Nicol, 2006). Birds with KBF are reported to have more difficulty with their mobility (Nasr et al., 2012), as they take longer to reach a food reward and approximately 4 times as long to fly from a perch to obtain a food reward compared to birds with no KBF.

As mobility becomes more difficult in the presence of these fractures, it has been suspected that this does lead to the onset of pain. For the first time evidence shown that hens with a KBF experience pain during an experiment using butorphanol treatment which when used improved the ability for a bird with a KBF to fly from a perch to the ground following treatment, while there was no effect of butorphanol on birds with no KBF (Nasr et al., 2012). In mammals, a bone fracture can be acutely painful (Maruyama et al., 2020). The presence of a fracture leads to an influx of inflammatory cells and inflammatory cytokines to the site of injury (Mountziaris & Mikos, 2008), including increased interleukin (IL)-1 gene expression in the brain of hens with KBF (Wei et al., 2019). As a bone fracture occurs, an inflammatory response will follow and often peaks within 24 hours in mammals (Mountziaris & Mikos, 2008). A cascade of inflammatory cytokines is elevated following the injury, including IL-1, IL-6 and tumor necrosis factor (TNF)-α which are reported to be essential for fracture healing through regulation of cell differentiation including osteoclasts. A study in mice found the absence of TNF-α impaired fracture healing (Wicaksana & Rachman, 2018). In addition, IL-1 increases within the first 24 hours and triggers the release of IL-6 before returning to baseline within 72 hours (Maruyama et al., 2020).

As a fracture occurs, serum corticosterone has been demonstrated to increase alongside the fracture in laying hens. A study which took laying hens with KBF and hens with no KBF observed increased serum corticosterone in the fracture group, which has been considered to be a sign of stress (Wei et al., 2019). That same study found the birds with KBF had elevated expression of inflammatory cytokines including IL-1β, TNF-α and nitric oxide producer nitric oxide synthesis (iNOS) in the brain, liver and abdominal tissue (Wei et al., 2019). Those authors draw the possibility that production of these cytokines might be related to bone repair and remodelling of the fracture site, similarly to in mammals which was observed by (Wicaksana & Rachman, 2018). However, the severity or age of the fractures was not clear from that study.

Stress has widely been associated with negative effects on the hippocampus and hippocampal dependent functions such as learning and memory (Kino, 2015; Robertson et al., 2017). In avian species there is emerging evidence that hens subject to stressors including unpredictable chronic mild stress (UCMS), feed restriction and heat stress all have negative effects on the hippocampus, including UCMS leading to reduced adult hippocampal neurogenesis (AHN) (Gualtieri et al., 2019; Robertson et al., 2017). However, these studies did not measure inflammatory cytokines in the brain, indicating no potential link between the onset of stress as a result from KBF and expression of inflammatory cytokines in the hippocampal formation (HF). There is however evidence from our laboratory of severe KBF leading to reduced AHN within the rostral and caudal HF and the onset of a depressive state (Armstrong et al., 2020). Hens with severe KBF were found to have lower densities of immature doublecortin (DCX) multipolar and bipolar neurons in the HF than hens with minimal KBF. Those authors also found a negative correlation between the fracture severity score at an individual bird level and DCX^+ cell density. There was also a relationship between the timespan of the fracture and density, where birds who acquired a fracture earlier in their lives had fewer DCX^+ multipolar neurons in the caudal HF (Armstrong et al., 2020). That study however did not investigate for any signs of inflammation which could have been upregulated as a result of KBF.

This chapter hypothesises that where DCX^+ is reduced in birds with severe KBF in comparison to those with minimal KBF, there will be increased gene expression of inflammatory cytokines in the HF. This chapter uses qPCR to measure gene expression of inflammatory cytokines associated with bone fracture, as well as microglia activation marker colony stimulating factor receptor-1 (CSFR-1) and brain derived neurotrophic factor (BDNF) in the rostral and caudal HF. Cytokines measured as part of this study include Inflammatory cytokines IL-1β, IL-6 and TNF-α, and anti-inflammatory cytokine IL-10. Expression will be compared amongst the minimal and severe KBF groups, as there were no birds with absent fractures. A correlation analysis will be performed between gene expression and the severity score, multipolar and bipolar density provided by Armstrong et al 2020, time point of the first fracture and time point of the final fracture as fractures were scored across 11 time points. The prediction for this study is that birds will respond to KBF with an inflammatory response in the hippocampus, based of previous findings in the avian species where KBF were associated with increased IL-1β and TNF-α in the brain. In addition, further prediction for this chapter is that there will be a negative correlation of inflammatory cytokines and neuron density, and a positive relationship with KBF severity score.

3.2 Methods

3.2.1 Ethical Statement

This experiment was granted ethical approval by the Animal Welfare and Ethical Review Body at Newcastle University, project ID AWERB 549.

3.2.2 Animals and housing

The samples used for this study are a follow on from a previous study in our lab, where full details can be found (Armstrong et al., 2020). In addition, the methodology described in this section regarding housing of the hens and identification of fractures, have been obtained from that same study.

The birds used in this chapter and by Armstrong et al 2020 were Lohman Brown (LB) chicks from the Aviforum research facility in Zollikofen, Switzerland. They were placed within eight mixed hybrid rearing pens at 1 day old. From 18 weeks, the hens were moved to a commercial aviary system which included an onsite layer barn. The layer barn consisted of 20 identical pens (450 x 700 x 230 cm) holding 225 hens in each, containing a lower, upper and nest box tier. Further details regarding housing can be found in our labs earlier study (Armstrong et al., 2020; Rufener, Baur, et al., 2018). Food and water were provided *ad libitum* through automatic feeding chains and nipple drinkers on the top and lower tier. Artificial light was present between 02:00 h until 16:00 h which included a dawn phase. Natural daylight was present through 08:00 till 16:00. Hens were able to access a range of perches including the top and lower tier enabling movement between tiers. Hens also had access to floor space covered in wood shavings which included below and beside the aviary. Pecking was encouraged through placement 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). Hens had access to further external space consisting of wood shavings and sand, known as the wintergarden. This space was accessible via automatically opening popholes, between 10:00 h and 16:30 h.

3.2.3 Data collection

Collection of 11 data points during different times of the production cycle which scored any present KBF's was performed originally for our lab's previous study (Armstrong et al., 2020). The way a KBF was scored is outlined in section 3.2.5. A pen of 20 hens were first obtained for data analysis every three weeks from week 21, two further pens were analysed from week 22.

3.2.4 Individual mobility

A custom made infrared (IR) tracking system validated by Rufener and colleagues 2018 using IR emitters recorded individual mobility for 6 days per time point. IR emitters were installed across various parts of the aviary, including the litter, lower tier, nest box, upper tier, and wintergarden. Focal hens had IR receivers which were mounted to their leg bands, to record the zone-specific signals produced by the IR emitters with a frequency of 1 Hz.

3.2.5 Keel bone assessment/fracture severity scoring

Radiographs were performed on day 8 after removal of the IR receiver, a mobile x-ray unit (GIERTH HF 200 ML; x-ray tube Toshiba D-124) at each data collection time point to detect any present KBF. Each hen received a total of 11 radiographs. This was at maximal acceleration voltage of 100 kV; x-ray plate Canon CXDI-50G; software Canon CXDI Control Software NE; distance: 80 cm, voltage: 46 kV/2.4 mAs) (Armstrong et al., 2020). The PACS system (picture Archiving and Communication System; IMPAZ EE, Agfa Healthcare, Bonn, Germany) of the Department of Clinical Radiology (Vetsuisse Faculty, University of Bern) as DICOM was used to analyse radiographs and determine the presence of a fracture and severity. A scale was used by Armstrong et al 2020 including a range from "no fracture" to "extremely severe", resulting in a continuous variable from 0.0 to 10.0, where 6.0 onwards was classed as a severe fracture. This method was adopted from Rufener et al 2018.

For the study in this chapter and the study prior to this by Armstrong et al 2020, there were no birds available where keel bone fractures were absent. This meant both studies were limited to minimal and severe fractures. However, Armstrong et al 2020 found there was a dramatic difference in DCX^+ staining of neuronal cells within the hippocampus between the minimal and severe fracture groups. Because of this, the severity score was used to determine a correlation between gene expression and severity score which will be described in section 3.3.3.

3.2.6 Tissue collection and processing

For the study carried out by Armstrong et al 2020, at no later than 4.5 weeks after the final X-ray, 24 hens were euthanised by an intravenous injection of pentobarbital (Esconarkon, 0.3ml/hen). Brains were removed from the skull and placed into 0.1M PBS in a Petri dish, then divided along the longitudinal fissure with a scalpel. For the purpose of this study, the rostral and caudal hippocampus from one hemisphere were available. These were preserved in RNAlater tissue storage reagent (Sigma-Aldrich, UK) for 48 hours at 4^{o}C, then frozen at -80^{o}C.

3.2.7 Quantitative real-time PCR

RNA was isolated from rostral and caudal hippocampal samples using methods described in the previous chapter in section 2.3.3. RNA was reverse transcribed again using the same method described in chapter 2. Gene specific primers were designed using the primer-BLAST tool (National Centre for Biotechnology Information), information regarding these primers can be found in section 2.3.4. This chapter measures the gene expression of inflammatory cytokines IL-1β, IL-6, TNF-α against housekeeping gene with lamin-b receptor (LBR) in a ratio and anti-inflammatory cytokine IL-10 to measure the ratio of pro-inflammatory and anti-inflammatory cytokine expression.

Additionally, microglia activation marker CSFR-1, BDNF and IL-10 will be analysed in ratio of gene expression to LBR. Full primer details including sequences can be found in the previous chapter in section 2.3.4 Preparation of standards, primer concentrations and RT-PCR conditions are outlined in the previous chapter in section 2.3.4.

3.2.8 Neuron Densities

Similar to the previous chapter, Doublecortin immunohistochemistry cell count data was provided by Elena Armstrong (Armstrong et al 2020), for rostral and caudal hippocampus samples obtained from the same brains (other hemisphere) used for PCR analysis. Cell counts were available for multipolar and bipolar neurons and a Z score (Zi) was performed to correct for differences between staining batches.

3.2.9 Statistical Analysis

This chapter used LBR as a reference gene where all target genes were compared in a ratio of target gene to reference gene, to normalise gene expression consistently with other studies (Dunn et al., 2013). For inflammatory cytokines IL-1β, TNF-α and IL-6, this was repeated with anti-inflammatory cytokine IL-10 in place of LBR. The log of each ratio was used to calculate a Z score (Zi), to account for potential variation between RT-PCR runs. The calculation for the Zi can be found in section 2.3.6. A Zi of DCX$^+$ multipolar and bipolar density in the HF was provided by Armstrong et al 2020 for use in this chapter. The severity of each fracture categorised as either minimal or severe based on the recorded severity score (1-5 minimal and 6-10 severe), was also provided by Armstrong et al 2020. There were three separate analyses of KBF severity including: minimal vs severe, the severity score and the time point the fracture was recorded. To determine differences in gene expression amongst birds with minimal vs severe fractures the LMER with type 3 ANOVA were used. This model was repeated to determine the correlation between the score of the fracture and gene expression, alongside the time point the fracture was first observed and last present with an increase of +1 in severity from the previous measurement. Finally, the LMER with type 3 ANOVA was repeated with the density of DCX+ multipolar and bipolar neurons and gene expression.

All graphs and statistical analysis are performed using R Studio (version 1.3.959). For analysis of rostral and caudal gene expression, the linear mixed effects model (LMER) with repeated measures and type 3 ANOVA was performed using the AFEX package. The Zi of multipolar and bipolar neuron density were both separately compared with the Zi of our target gene expression to determine any correlation, using again the LMER and type 3 ANOVA. For this chapter, the statistical model included rostral and caudal HF as within subject factors and fracture group as fixed effect. The fracture severity score and time point a fracture was first and last observed were included as random effects. Analysis included the correlation between the age of the fracture, severity score of the fracture and DCX$^+$ density with the expression of all genes measured in the HF as three separate analyses. The age of the fracture was analysed in two analyses consisting of when the fracture was first and last present before sampling. In addition, DCX$^+$ density correlated with gene expression in the HF of birds in categories of minimal and severe fractures was performed along with a direct analyses of gene expression in the HF of birds with minimal and severe fractures.

Where a significant interaction or main effect was found ($p<0.05$), a pairwise comparison was performed using the estimated marginal means (EMMEANS) package. Statistical significances of $p<0.05$ are listed in text where appropriate, full statistical analysis can be found in appendix 2. All graphs were produced using GGPLOT2 in R Studio where the natural log from each ratio of gene expression has been plotted unless specified.

3.3 Results

3.3.1 Gene expression

There were no significant differences in any of the genes between the severe and minimal KBF groups, nor between the rostral and caudal HF, except for IL-10/LBR and BDNF/LBR (table 8).

There was a significant two-way interaction between fracture group and HF subdivision for IL-10/LBR expression. Pair-wise analyses showed that in birds with minimal KBF only, there is a significant difference in IL-10 expression between the rostral and caudal HF where expression was greater in the caudal ($t(19)=2.910$, $P=0.0404$). However, in the rostral HF only, there is a difference in gene expression between fracture groups where IL-10/LBR is expressed in larger quantities in birds with severe KBF than minimal KBF ($t(37)=-3.187$, $P=0.0148$) (table 9, figure 7). Fracture group does not change BDNF/LBR gene expression, but BDNF expression is greater in the rostral than caudal HF across all birds ($F(1,20)=9.6441$, $P=0.005441$) (table 9, figure 10).

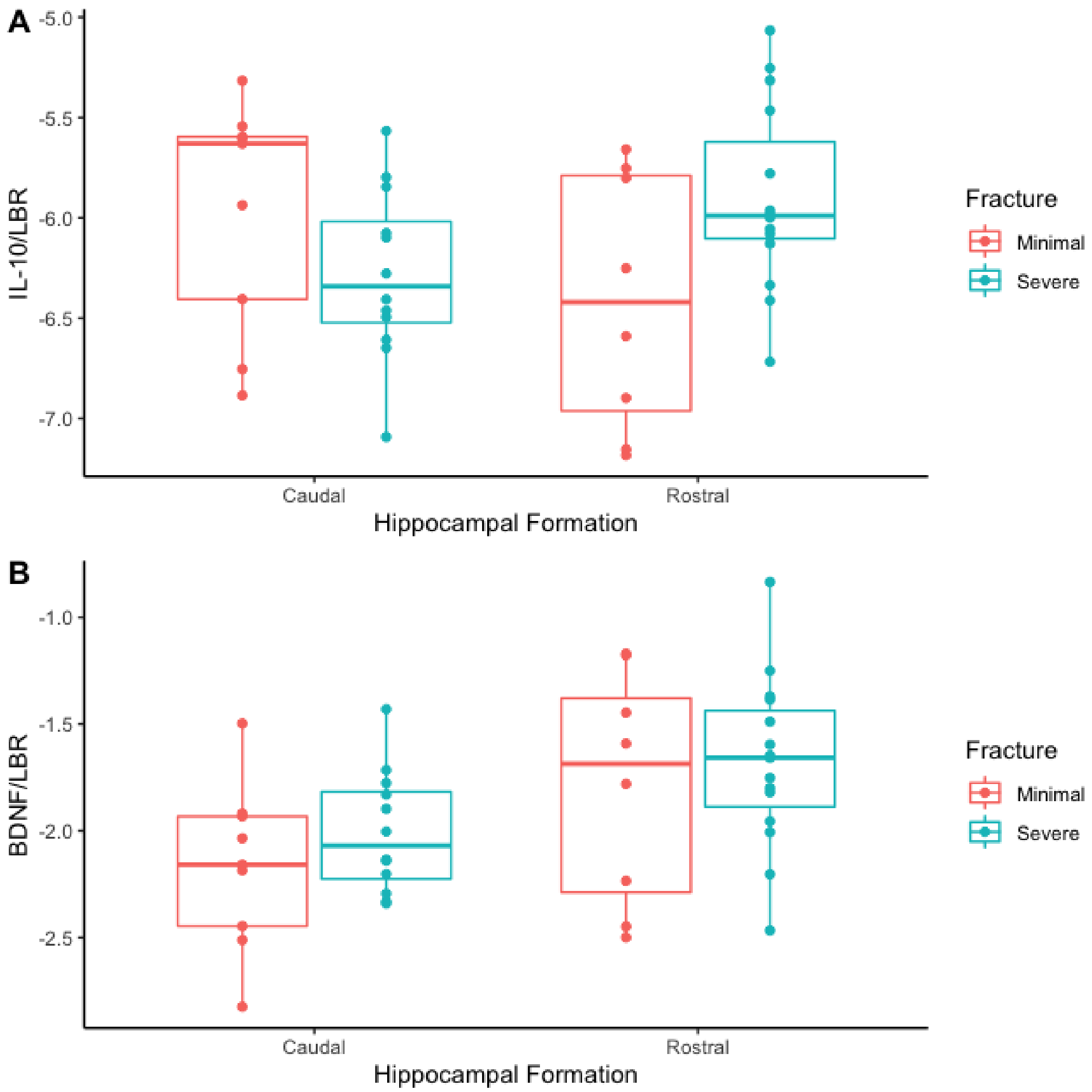

Figure 9: IL-10/LBR gene expression is increased in the caudal HF of birds with minimal KBR and BDNF/LBR gene expression is increased in the rostral HF of all birds.

The ratio of IL-10/LBR (A) and BDNF/LBR (B) gene expression in the rostral or caudal hippocampus of birds with either minimal or severe keel bone fractures. The line visualises the median of each group with the hinges representing the upper and third quartile. Sample n= Severe: 15 rostral 12 caudal, Minimal: 8 rostral 9 caudal.

Table 8: Gene expression in the rostral and caudal HF of birds with minimal and severe KBF

Statistical output from the linear mixed model with three-way ANOVA of gene expression in the rostral and caudal HF of birds with minimal or severe KBF. All significant interactions (p<0.05) are highlighted in bold.

IL-1β/LBR	Fracture	F(1,20)=0.0057, P=0.9404
	HF	F(1,20)=0.9053, P=0.3524
	Fracture : HF	F(1,20)=0.0924, P=0.7642
IL-1β/IL-10	Fracture	F(1,20)=0.4662, P=0.5023
	HF	F(1,20)=1.1231, P=0.3015
	Fracture : HF	F(1,20)=0.9231, P=0.3478
IL-6/LBR	Fracture	F(1,21)=2.2381, P=0.1492
	HF	F(1,20)=1.1944, P=0.2871
	Fracture : HF	F(1,20)=1.1505, P=0.2959
IL-6/IL-10	Fracture	F(1,21)=0.0070, P=0.93422
	HF	F(1,20)=0.2738, P=0.60637
	Fracture : HF	F(1,20)=4.0723, P=0.05682 .
TNF-α/LBR	Fracture	F(1,20)=0.3774, P=0.5456
	HF	F(1,20)=1.3539, P=0.2581
	Fracture : HF	F(1,20)=0.0148, P=0.9044
TNF-α/IL-10	Fracture	F(1,20)=0.0762, P=0.7852
	HF	F(1,20)=0.7174, P=0.4068
	Fracture : HF	F(1,20)=2.3099, P=0.1438
IL-10/LBR	Fracture	F(1,21)=1.4917, P=0.235312
	HF	F(1,19)=0.6582, P=0.426853
	Fracture : HF	**F(1,19)=13.8315, P= 0.001377**
BDNF/LBR	Fracture	F(1,20)=0.8396, P=0.370112
	HF	**F(1,20)=9.6441, P=0.005441**
	Fracture : HF	F(1,20)=0.1158, P=0.737098
CSFR-1/LBR	Fracture	F(1,21)=0.0830, P=0.7760
	HF	F(1,20)=2.7685, P=0.1114
	Fracture : HF	F(1,20)=0.5982, P=0.4482

Table 9: Estimated marginal means of gene expression in the rostral and caudal HF of birds with severe and minimal KBF

Pair-wise analysis of the estimated marginal means (EMMEANS) of all significant interactions between gene expression in the rostral and caudal HF of birds with minimal or severe KBF. Significant interactions (p<0.05) are highlighted in bold with the difference between those treatment groups listed.

IL-10/LBR	Minimal Caudal : Severe Caudal	t(38)= 1.197, P=0.6328	-
	Minimal Caudal : Minimal Rostral	**t(19)= 2.910, P=0.0404**	**-Caudal increase**
	Minimal Caudal : Severe Rostral	t(36)= -0.531, P=0.9509	-
	Severe Caudal : Minimal Rostral	t(38)= 1.411, P=0.5002	-
	Severe Caudal : Severe Rostral	t(20)= -2.316, P=0.1272	-
	Minimal Rostral : Severe Rostral	**t(37)= -3.187, P=0.0148**	**-Severe rostral increase**
BDNF/LBR	**Caudal : Rostral**	**t(21)= -3.106, P=0.0054**	**-Rostral increase**

3.3.2 Neuron densities

As a next step I ran a correlation analysis between the gene expression in the HF and the density of multipolar and bipolar neuron density. There was no significant relationship between multipolar and bipolar DCX$^+$ density and any of the genes measured in the rostral and caudal HF, indicating at the time samples were obtained and DCX$^+$ was reduced there was no presence of increased cytokine gene expression (table 13, table 14).

3.3.3 Keel bone fracture severity score

Next, a correlation between the severity score of the KBF and gene expression in the rostral and caudal HF was made (table 10). There was a significant interaction between severity score and HF subdivision on BDNF/LBR expression (F(1,17)=5.0076, P=0.03873). The slope of BDNF/LBR gene expression was shallower in the rostral HF than caudal however, neither of these slopes were individually significantly different from zero (figure 11A).

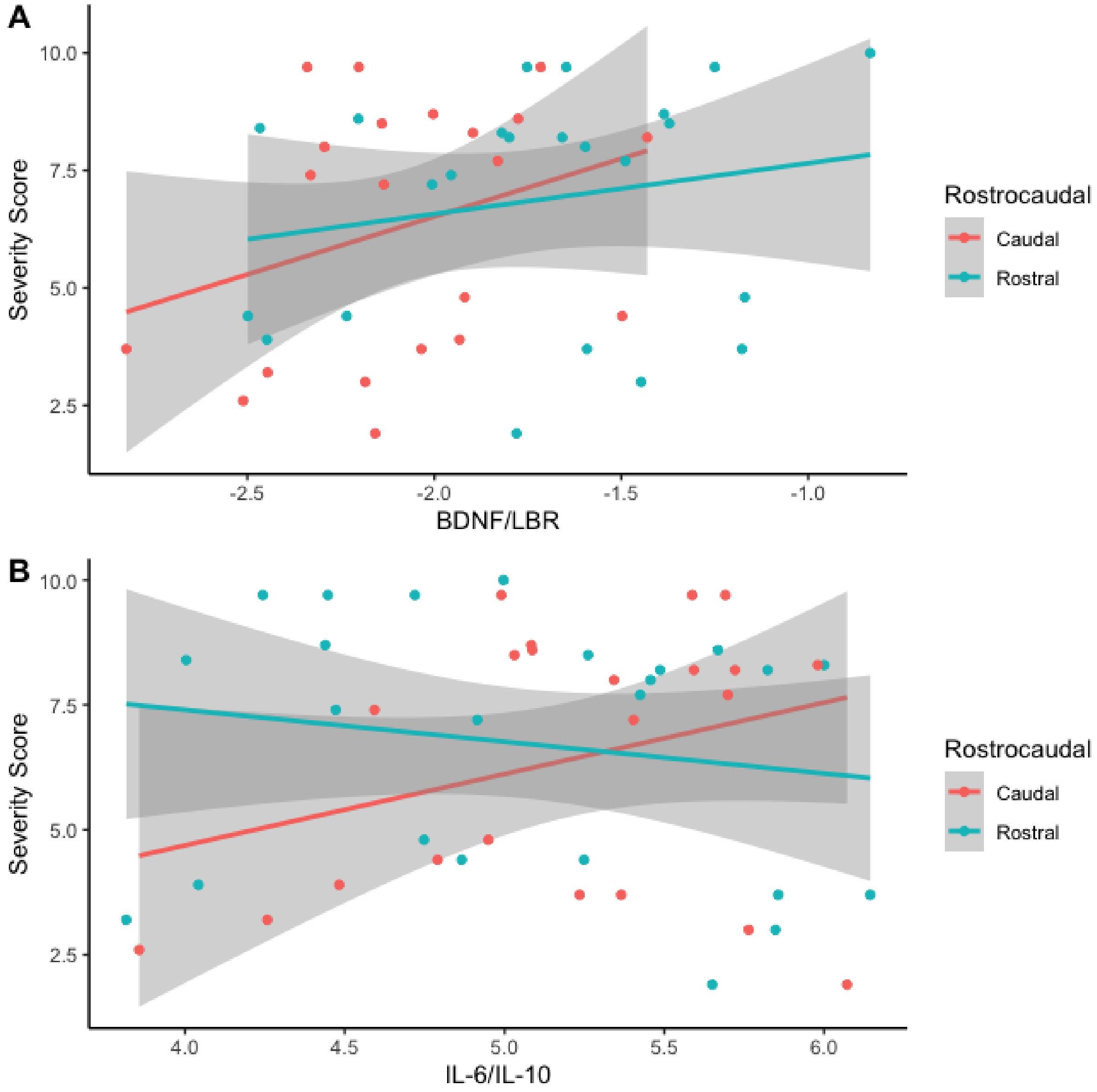

Figure 10: Correlation between fracture severity score and gene expression of BDNF/LBR and IL-6/IL-10 in the rostral and caudal HF

Correlation between the KBF severity score matched with the ratio log of BDNF/LBR (A) and IL-6/IL-10 (B) gene expression in the rostral and caudal HF of each bird with KBF. Shading representative of standard error of the slope.

A: Sample n= 23 rostral, 21 caudal.
B: Sample n= 24 rostral, 22 caudal.

Table 10: Correlation between KBF severity score and gene expression in the rostral and caudal HF

Statistical output from the linear mixed model with three-way ANOVA of the correlation between individual KBF severity score and gene expression in the rostral and caudal HF of birds with minimal or severe KBF. All significant interactions (p<0.05) are highlighted in bold.

KBF severity score	IL-1β/LBR	F(1,18)=2.3146, P=0.1454
	HF	F(1,18)=0.8374, P=0.3722
	IL-1β/LBR : HF	F(1,18)=0.6716, P=0.4231
KBF severity score	IL-1β/IL-10	F(1,18)=0.3926, P=0.5388
	HF	F(1,18)=1.4236, P=0.2483
	IL-1β/IL-10 : HF	F(1,18)=2.8410, P=0.1090
KBF severity score	IL-6/LBR	F(1,19)=0.1094, P=0.74438
	HF	F(1,19)=1.6486, P=0.21457
	IL-6/LBR : HF	F(1,19)=3.5033, P=0.07668 .
KBF severity score	IL-6/IL-10	F(1,19)=4.0823, P=0.05746 .
	HF	F(1,19)=3.4946, P=0.07705 .
	IL-6/IL-10 : HF	**F(1,19)=6.5571, P=0.01909**
KBF severity score	TNF-α/LBR	F(1,18)=0.5617, P=0.4632
	HF	F(1,18)=1.1340, P=0.3010
	TNF-α/LBR : HF	F(1,18)=0.0354, P=0.8529
KBF severity score	TNF-α/IL-10	F(1,18)=0.0606, P=0.8084
	HF	F(1,18)=1.6977, P=0.2090
	TNF-α/IL-10 : HF	F(1,18)=0.5395, P=0.4720
KBF severity score	IL-10/LBR	F(1,17)=2.7850, P=0.1133
	HF	F(1,17)=2.3850, P=0.1409
	IL-10/LBR : HF	F(1,17)=1.9428, P=0.1812
KBF severity score	BDNF/LBR	F(1,17)=0.6984, P=0.41482
	HF	F(1,17)=3.0120, P=0.10069
	BDNF/LBR : HF	**F(1,17)=5.0076, P=0.03873**
KBF severity score	CSFR-1/LBR	F(1,19)=0.2188, P=0.6451
	HF	F(1,19)=1.1155, P=0.3041
	CSFR-1/LBR : HF	F(1,19)=0.1112, P=0.7425

Similarly to BDNF there as a significant interaction between severity score and HF subdivision on IL-6/IL-10 gene expression (F(1,19)=6.5571, P=0.01909), where gene expression increases in the caudal HF, and decreases in the rostral HF as fracture severity increases (figure 11). However, this trend was not significant for each HF subdivision alone suggesting KBF severity does not predict expression of either gene across the whole HF.

To determine if the age of the fracture impacts gene expression, a correlation analysis was performed (table 11, table 12). The timing of the first fracture interacted significantly with HF subdivision for the expression of IL-1β/LBR (F(1,21)=4.8656, P=0.03854), IL-1β/IL-10 (F(1,21)=9.5471, P=0.005533), IL-6/IL-10 (F(1,21)=4.9862, P=0.03646), and IL-10/LBR (F(1,20)=5.5705, P=0.02835). They also all vary across the HF subdivisions. It appears that we see opposing effects between the rostral and caudal HF. Expression of IL-1β/LBR (F(1,19)=9.25, P=0.006) and IL-1β/IL-10 (F(1,19)=9.58, P=0.005) significantly increased in the rostral HF when KBF were acquired earlier, where IL-10/LBR decreases (F(1,21)=5.55, P=0.028). There were no significant trends in the caudal HF and even though there was a significant interaction between the HF subdivision for IL-6/IL-10 there was no significant trends between time of fracture and individual expression in the rostral or caudal HF (figure 12).

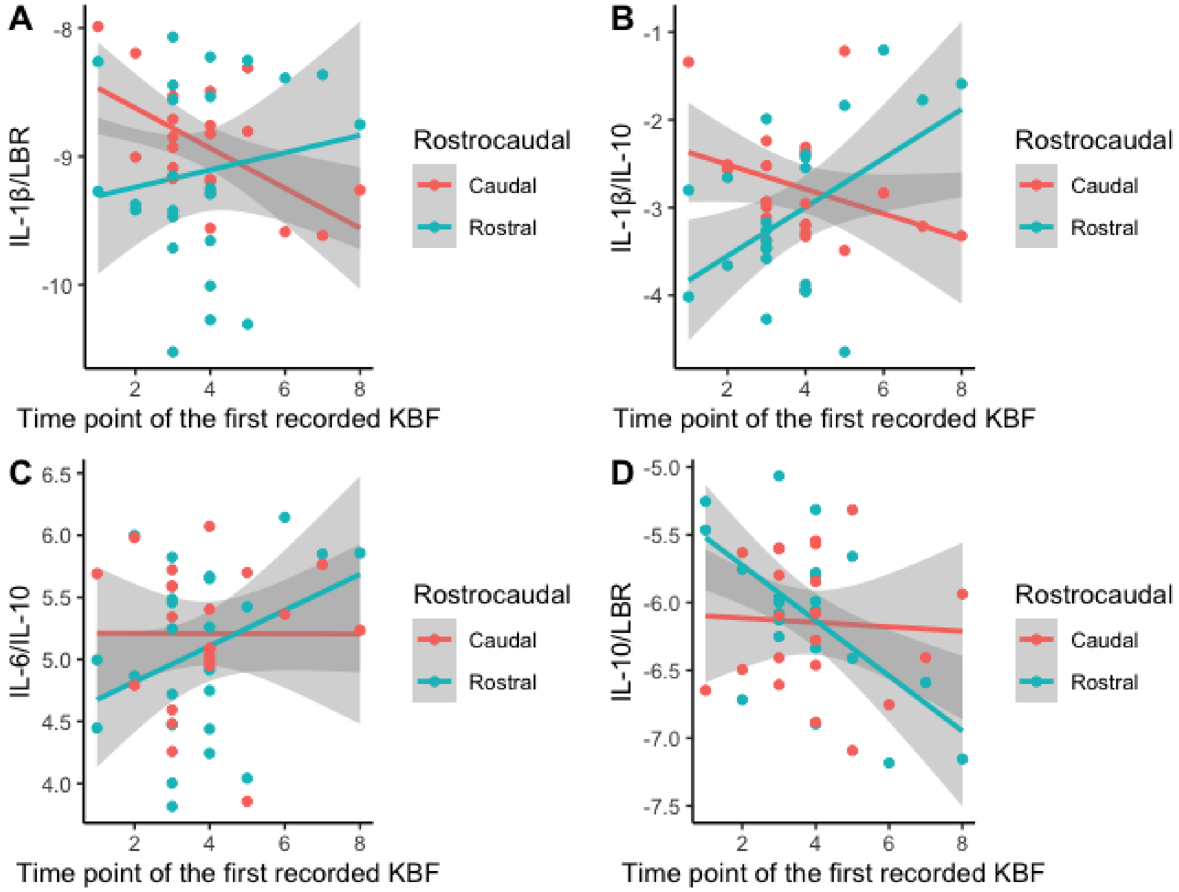

Figure 11: Correlation between gene expression of IL-1β/LBR, IL-1β/IL-10, IL-6/IL-10 and IL-10/LBR in the rostral and caudal HF with the time point a KBF was first recorded.

Correlation between gene expression ratio log of IL-1β/LBR (A), IL-1β/IL-10 (B), IL-6/IL-10 (C) and IL-10/LBR (D) and the time point of the first recorded severity score in the rostral and caudal HF. Shading representative of standard error of the slope.

A+B: Sample n= 23 rostral 21 caudal.
C: Sample n= 24 rostral 22 caudal.
D: Sample n= 21 rostral 24 caudal.

There appears to be no statistical relationship between the earlier the fracture being recorded and expression of the remaining genes (table 11), indicating that older fractures don’t lead to more or less gene expression of these cytokines.

Table 11: Correlation between the time point a fracture was first recorded and gene expression in the rostral and caudal HF

Statistical output from the linear mixed model with three-way ANOVA of the correlation between gene expression in the rostral and caudal HF and the time point of the first recorded severity score of a KBF of birds with minimal or severe KBF. All significant interactions (p<0.05) are highlighted in bold.

IL-1β/LBR	First score	F(1,21)=0.6759, P=0.42002
	HF	**F(1,22)=6.2022, P=0.02073**
	First score : HF	**F(1,21)=4.8656, P=0.03854**
IL-1β/IL-10	First score	F(1,21)=0.0257, P=0.874128
	HF	**F(1,21)=11.8286, P=0.00234**
	First score : HF	**F(1,21)=9.5471, P=0.005533**
IL-6/LBR	First score	F(1,22)=1.5082, P=0.2322
	HF	F(1,21)=0.1863, P=0.6702
	First score : HF	F(2,21)=0.7042, P=0.4107
IL-6/IL-10	First score	F(1,22)=0.0682, P=0.79634
	HF	**F(1,21)=5.8632, P=0.02431**
	First score : HF	**F(1,21)=4.9862, P=0.03646**
TNF-α/LBR	First score	F(1,23)=0.0001, P=0.9916
	HF	F(1,23)=0.1030, P=0.7511
	First score : HF	F(1,22)=0.0125, P=0.9120
TNF-α/IL-10	First score	F(1,23)=0.4393, P=0.5139
	HF	F(1,24)=1.4179, P=0.2453
	First score : HF	F(1,23)=0.7580, P=0.3929
IL-10/LBR	First score	F(1,21)=1.8018, P=0.19376
	HF	**F(1,21)=4.7489, P=0.04058**
	First score : HF	**F(1,20)=5.5705, P=0.02835**
BDNF/LBR	First score	F(1,20)=0.0047, P=0.9458
	HF	F(1,21)=0.0328, P=0.8579
	First score : HF	F(1,20)=2.4894, P=0.1297
CSFR-1/LBR	First score	F(1,22)=0.8097, P=0.3778
	HF	F(1,21)=1.6972, P=0.2065
	First score : HF	F(1,20)=0.4469, P=0.5111

Finally, a correlation analysis between the time a fracture was last present (table 12). show was a significant two-way interaction between time since the last recorded fracture and HF subdivision on IL-6/IL-10 expression (F(1,20)=13.3386, P=0.00149). It appears that the later the fracture was reported representing newer fractures, the greater IL-6/IL-10 expression is in the rostral HF (F(1,22)=6.05, P=0.022) which could point toward an initial involvement of IL-6 when a KBF first arises. In the caudal HF we see a non-significant trend in the opposite direction (figure 13).

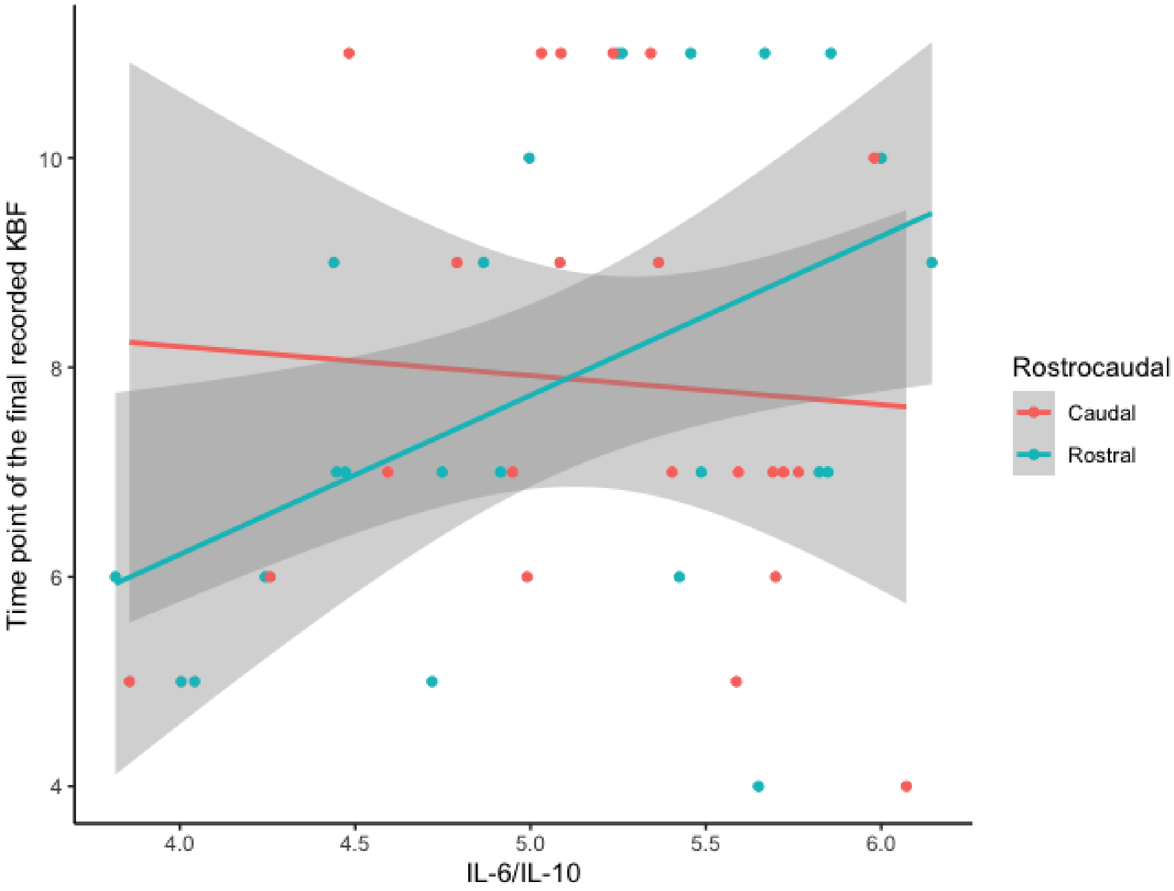

Figure 12: Correlation between gene expression of IL-6/IL-10 in the rostral and caudal HF and the time point of the final recorded fracture.

Correlation between gene expression of IL-6/IL-10 ratio log and the time point of the last recorded severity score in the rostral and caudal HF. Shading representative of standard error of the slope. Sample n= 24 rostral 22 caudal.

As of the remaining genes measured, the later the fracture was recorded potentially representing newer fractures, expression of inflammatory cytokines was uncorrelated (table 12).

Table 12: Correlation between the time point a fracture was last recorded and gene expression in the rostral and caudal HF

Statistical output from the linear mixed model with three-way ANOVA of the correlation between gene expression in the rostral and caudal HF and the time point of the last recorded severity score of a KBF (increase of +1 point from the previous scoring) of birds with minimal or severe KBF. All significant interactions (p<0.05) are highlighted in bold.

IL-1β/LBR	Last score	F(1,21)=0.6596, P=0.4258
	HF	F(1,21)=0.0093, P=0.9241
	Last score: HF	F(1,21)=0.0390, P=0.8454
IL-1β/IL-10	Last score	F(1,21)=0.0367, P=0.8499
	HF	F(1,21)=1.5769, P=0.2229
	Last score : HF	F(1,21)=0.8794, P=0.3590
IL-6/LBR	Last score	F(1,22)=1.7776, P=0.1960
	HF	F(1,21)=2.3581, P=0.1395
	Last score : HF	F(1,21)=1.8374, P=0.1896
IL-6/IL-10	Last score	F(1,22)=1.1491, P=0.295300
	HF	**F(1,20)=14.4939, P=0.00103**
	Last score : HF	**F(1,20)=13.3386, P=0.00149**
TNF-α/LBR	Last score	F(1,21)=1.2891, P=0.2688
	HF	F(1,20)=0.7607, P=0.3930
	Last score : HF	F(1,20)=1.5762, P=0.2233
TNF-α/IL-10	Last score	F(1,21)=0.4232, P=0.5224
	HF	F(1,21)=0.1028, P=0.7517
	Last score : HF	F(1,20)=0.0003, P=0.9863
IL-10/LBR	Last score	F(1,20)=0.2244, P= 0.64063
	HF	F(1,20)=4.1027, P=0.05595
	Last score : HF	F(1,20)=4.3183, P=0.05067
BDNF/LBR	Last score	F(1,20)=1.0115, P=0.3262
	HF	F(1,21)=0.3740, P=0.5474
	Last score : HF	F(1,20)=0.0697, P=0.7944
CSFR-1/LBR	Last score	F(1,22)=0.2795, P=0.6023
	HF	F(1,20)=1.2521, P=0.2760
	Last score : HF	F(1,20)=0.4071, P=0.5305

3.4 Discussion

The data presented in this chapter comes as a follow up to earlier work by Armstrong 2020 who determined birds with severe KBF had lower DCX$^+$ neuron density than birds with minimal KBF. This chapter hypothesised that inflammatory related gene expression would be increased in the rostral and caudal HF of laying hens with severe KBF, which was not the case.

Results showed whilst there were no differences in gene expression for the vast majority of genes, there was a two-way interaction of IL-10/LBR expression between fracture group and HF subdivision, affecting the rostral HF (table 8, figure 10). There was no relationship between neuron density and gene expression, but there was significant trends in the time since the first fracture was acquired and IL-1β, IL-10 and IL-6 gene expression in the rostral HF. Birds who acquired KBF earlier tend to have more IL-1β and less IL-10 in the rostral HF, whereas birds who acquired KBF later had increased IL-6.

In mammals, the hippocampus is split across two sub-regions: the ventral which is involved with emotion, stress and affect and the dorsal which regulates cognitive functions (Greene & Pisano, 2012a). In birds, the HF is split into the caudal and rostral regions which their specific roles and biological processes are less well known making the significance of this result more difficult to interpret (Smulders, 2017). However a study by (Gualtieri et al., 2019) used a UCMS protocol to look at neurogenesis in the caudal HF in comparison to the ventral in mammals. Results shown the caudal pole of the laying hen is particularly sensitive to UCMS followed by a reduction in the density of DCX$^+$ multipolar neurons and a non-significant reduction of bipolar cells. This result was strengthened by reduced baseline CORT titres suggesting adjustment of the stress response and reduced spleen mass. Overall (Gualtieri et al., 2019) used UCMS to demonstrate a stress-led reduction in neurogenesis in the caudal HF and considered the caudal HF to be homologous to the ventral HF in rodents, which their roles in stress regulation were consistent with one another. This study found a decrease in BDNF gene expression in the caudal HF in all birds, irrespective of fracture severity. Unfortunately, there were no birds with absent fractures to compare against, it is unclear whether if the findings from this chapter would be consistent if this study included birds with absent fractures or if samples were obtained shortly after a fracture was found. However, overall expression of BDNF expression can differ between anatomical regions under normal circumstances in mammalian species (Bathina & Das, 2015), which could also apply to the avian HF based on findings in this chapter.

To determine if fracture is a possible cause of BDNF downregulation in the hippocampus, a correlation analysis between BDNF expression in both caudal and rostral was performed with the individual severity score of fracture from each bird. Results in figure 3 show that whilst expression of BDNF is statistically different between rostral and caudal HF, expression increases with a higher severity score in both. It remains unlikely BDNF is a mediator for the downregulation in neurogenesis in these birds found by (Armstrong et al., 2020). In this study and the study in chapter 2, samples for analysis were obtained on one occasion when hens had present KBF for a long period of time and birds were already in poor physical condition. Future work should include analysis of cytokines at different time points following any signs of chronic stress, for example shortly after a fracture is observed. Overall, the results presented in this study and prior study open the possibility that if cytokines are involved with reduced hippocampal plasticity, they are likely to be involved prior to when samples were obtained. Therefore, it would be useful for future studies to obtain samples from multiple time points during chronic stress, or even when fractures first occur to investigate expression of these cytokines.

As previously mentioned, all birds within this study had some level of KBF and there were no controls in place for selecting birds with either new or old fractures, however, fractures were measured across 11 time points across the production cycle. It could be the case that some birds had newer fractures where pain might be acute and the inflammatory response is only beginning, or they could be old fractures and healing to some level, which could explain why IL-10 increases with increasing severity in the caudal HF. To account for this, a correlation analysis was run with all genes and the time point of the first recorded fracture and last. Results show that the ratio of IL-1β/IL-10 and IL-1β/LBR was significantly increased in the rostral HF of birds who acquired KBF earlier, with the reverse pattern for IL-10/LBR. In addition, expression of IL-6/IL-10 was increased in the rostral HF of birds who acquired KBF later in the study. These effects were limited to the rostral HF, with no relationship between the timing of the fracture and gene expression in the caudal HF (although expression does tend to point in the opposing direction whilst unsignificant). There was no effect of severity score on any genes including IL-10 and as IL-10 is increased in birds with minimal fractures compared to birds with severe fractures in the caudal HF (figure 11), the meaning of this is unclear. Increased IL-6 expression in the hippocampus with younger fractures indicates the presence of inflammation and fits with findings in rodents where systemic IL-6 is increased during wound healing demonstrating an importance of this cytokine (Maruyama et al., 2020) , however this was not in the hippocampus and was measured as a systemic immune response. As the present study did not explore systemic expression, it is unclear if this IL-6 expression is systemic first or local to the hippocampus. In addition, (Wei et al., 2019) discovered various other cytokines including IL-1β and TNF-α which were unchanged for this study were increased in the brain of birds with severe KBF, although this study measured whole brain RNA and used Lohman White birds opposed to Lohman Brown in the present chapter. Wei et al 2019 did not report or measure any changes in expression of IL-6, so it could be possible that a difference in IL-6 expression in birds without vs with KBF might have been present in the present study if those birds were available. Although one key difference between those two studies is that in line with the present study (Armstrong et al., 2020) looked at the effect of fractures on two specific areas of the HF: rostral and caudal, whereas Wei et al 2019 measured gene expression in the whole brain.

As previously mentioned (Armstrong et al., 2020) found that whilst the overall level of DCX^+ neuron density was decreased across the HF in birds with minimal fractures, it was also discovered that birds who had fractures occurring earlier had fewer multipolar DCX^+ density in the caudal HF. This finding by Armstrong et al 2020 alongside previously mentioned study in KBF with increased IL-1β in the whole brain (Wei et al., 2019) potentially indicate some involvement of IL-1β in mediating AHN in birds with older KBF. However, the limitations of no birds without a fracture might explain why there was no overall statistical difference in expression amongst minimal vs severe (table 8).

The reduction in AHN in birds with severe KBF was not limited to just the caudal HF, in fact Armstrong et al 2020 found a reduction of multipolar and bipolar neurons in the rostral as well. However, the reduction was reported to be stronger in the caudal HF. This could indicate a correlation between genes IL-1β and IL-10 and time since the last fracture in the rostral HF. The birds used for this experiment were obtained at the 11th time point of fracture scoring meaning if there was an extended period of time between fracture occurrence and sampling, cytokine expression might have reverted to baseline levels. This can explain why when relating fracture occurrence to IL-10 expression, IL-10 expression in the rostral HF is increased in birds who had fractures earlier. Together these results are consistent with other studies where IL-1b has been observed to be secreted following IL-6 in response to a fracture (Maruyama et al., 2020). It would make sense that in birds who had acquired more recent fractures we are seeing increased IL-6 and IL-1β in the rostral HF alongside decreased IL-10. Finally, when relating gene expression to the time point at which a fracture was last present, when fractures were present closer to sampling there was increased IL-6 in the rostral HF.

As a next step, it will be important to investigate the stages of the immune response over multiple time points during chronic stress. As there is emerging evidence from this study where IL-1β expression increases during the early stages of fracture in the hippocampus, at the time these samples were obtained, there could be other mechanisms involved such as microglia cells. Microglia cells reside in the HF and have been previously linked with neurogenesis, however these cells remain active whilst in either their M1/ameboid inflammatory state, or their M2/ramified neuroprotective state (Pérez-Domínguez et al., 2017; Zhou et al., 2017; Olmos & Lladó, 2014). As it appears IL-6 and IL-1β expression increases in the rostral HF in birds with more recent fractures and IL-10 increased in birds with older fractures which have potentially healed, although unchanged CSFR-1 expression (which only measures microglia activation) leaves the possibility of M1 inflammatory microglia involvement in response to IL-1β and IL-6 expression point towards further study into microglia in the HF.

3.5 Concluding remarks

To conclude, this chapter shows a potential involvement of inflammatory cytokines in the HF of birds with keel bone fractures, however as there were no birds with absent fractures available, this study’s findings remain limited. Future work should include birds with absent fractures in direct comparison with fractures to strengthen these findings. Furthermore, this study was not able to measure gene expression when a fracture first occurs which would provide greater opportunity to explore cytokine expression in the HF. As there were no differences in expression of inflammatory cytokines in birds with minimal vs severe KBF, expression was correlated with the fracture severity score and how recent or new the KBF occurred to the final sampling point. Results shown that expression of none of the genes measured correlated with DCX$^+$ neuron density across the HF, neither did the severity of the fracture influence expression. However, this study did find hens who acquired their initial KBF more recently had increased IL-1β and decreased IL-10 and those who acquired their last fractures more recently had increased IL-6 in the rostral HF only. Furthermore, hens with severe KBF had increased IL-10 in the rostral HF compared to the minimal fracture group. These results point toward a potential role of inflammatory cytokines in the rostral HF of hens with KBF, suggesting their involvement with chronic stress. However, their role in reducing hippocampal plasticity is not clear suggesting further study into inflammatory markers including microglia.

3.6 Appendix 2

Table 13: Correlation between Multipolar DCX+ density and gene expression in the HF

Statistical output from the linear mixed model with three-way ANOVA of the correlation between Multipolar DCX$^+$ density and gene expression in the rostral and caudal HF of birds with minimal or severe KBF. All significant interactions ($p<0.05$) are highlighted in bold.

Multipolar DCX$^+$ Density	IL-1β/LBR	F(1,27)=1.1920, P=0.2843
	HF	F(1,19)=0.7173, P=0.4072
	IL-1β/LBR : HF	F(1,31)=0.2043, P=0.6544
Multipolar DCX$^+$ Density	IL-1β/IL-10	F(1,26)=0.9155, P=0.3473
	HF	F(1,19)=0.7269, P=0.4041
	IL-1β/IL-10 : HF	F(1,30)=0.0818, P=0.7768
Multipolar DCX$^+$ Density	IL-6/LBR	F(1,40)=0.7707, P=0.3852
	HF	F(1,20)=0.3879, P=0.5403
	IL-6/LBR : HF	F(1,23)=0.0043, P=0.9483
Multipolar DCX$^+$ Density	IL-6/IL-10	F(1,37)=1.0236, P=0.3181
	HF	F(1,20)=0.4242, P=0.5222
	IL-6/IL-10 : HF	F(1,23)=0.0079, P=0.9299
Multipolar DCX$^+$ Density	TNF-α/LBR	F(1,33)=0.9733, P=0.3310
	HF	F(1,19)=0.8693, P=0.3623
	TNF-α/LBR : HF	F(1,25)=0.1558, P=0.6963
Multipolar DCX$^+$ Density	TNF-α/IL-10	F(1,28)=1.2618, P=0.2706

	HF TNF-α/IL-10 : HF	F(1,18)=0.7372, P=0.4013 F(1,26)=0.0325, P=0.8582
Multipolar DCX$^+$ Density	IL-10/LBR HF IL-10/LBR : HF	F(1,30)=0.3650, P=0.5501 F(1,19)=0.3729, P=0.5486 F(1,27)=0.0599, P=0.8085
Multipolar DCX$^+$ Density	BDNF/LBR HF BDNF/LBR : HF	F(1,39)=0.4407, P=0.5107 F(1,27)=0.0035, P=0.9533 F(1,37)=0.7120, P=0.4041
Multipolar DCX$^+$ Density	CSFR-1/LBR HF CSFR-1/LBR : HF	F(1,41)=1.7496, P=0.1931 F(1,21)=0.8333, P=0.3716 F(1,22)=0.9505, P=0.3400

Table 14: Correlation between Bipolar DCX+ density and gene expression in the HF

Statistical output from the linear mixed model with three-way ANOVA of the correlation between Bipolar DCX$^+$ *density and gene expression in the rostral and caudal HF of birds with minimal or severe KBF. All significant interactions (p<0.05) are highlighted in bold.*

Bipolar DCX$^+$ Density	IL-1β/LBR	F(1,31)=3.6317, P=0.06598
	HF	F(1,20)=0.7370, P=0.40070
	IL-1β/LBR : HF	F(1,34)=0.0015, P=0.96907
Bipolar DCX$^+$ Density	IL-1β/IL-10	F(1,31)=1.8111, P=0.1881
	HF	F(1,20)=0.6908, P=0.4155
	IL-1β/IL-10 : HF	F(1,35)=0.0210, P=0.8857
Bipolar DCX$^+$ Density	IL-6/LBR	F(1,41)=0.2905, P=0.5928
	HF	F(1,20)=1.3338, P=0.2612
	IL-6/LBR : HF	F(1,27)=0.3795, P=0.5429
Bipolar DCX$^+$ Density	IL-6/IL-10	F(1,41)=0.0076, P=0.9308
	HF	F(1,20)=1.5350, P=0.2292
	IL-6/IL-10 : HF	F(1,28)=0.0327, P=0.8578
Bipolar DCX$^+$ Density	TNF-α/LBR	F(1,39)=0.7387, P=0.3953
	HF	F(1,20)=0.6887, P=0.4161
	TNF-α/LBR : HF	F(1,30)=0.0379, P=0.8470
Bipolar DCX$^+$ Density	TNF-α/IL-10	F(1,38)=0.3415, P=0.5624
	HF	F(1,20)=0.7880, P=0.3853
	TNF-α/IL-10 : HF	F(1,36)=0.2668, P=0.6086
Bipolar DCX$^+$ Density	IL-10/LBR	F(1,40)=0.0102, P=0.9201
	HF	F(1,20)=1.0184, P=0.3248
	IL-10/LBR : HF	F(1,37)=1.3961, P=0.2448
Bipolar DCX$^+$ Density	BDNF/LBR	F(1,38)=0.3683, P=0.5475
	HF	F(1,27)=0.0181, P=0.8939
	BDNF/LBR : HF	F(1,38)=0.0420, P=0.8387
Bipolar DCX$^+$ Density	CSFR-1/LBR	F(1,38)=0.9642, P=0.3323
	HF	F(1,21)=0.9717, P=0.3351
	CSFR-1/LBR : HF	F(1,25)=0.7932, P=0.3814

CHAPTER 4: DOES THE MORPHOLOGY OF MICROGLIA DIFFER BETWEEN HENS WITH MINIMAL AND SEVERE KEEL BONE FRACTURES?

4.1 Abstract

Hens with severe keel bone fractures (KBF) show reduced neuronal plasticity in the rostral and caudal HF. The reduced plasticity of neurons is considered to be triggered by chronic stress which is a consequence of KBF. This chapter explores the potential role of microglia in the HF of hens with minimal and severe KBF, by counting cell density of microglia using the microglial marker IBA-1^+ in the medial and lateral wall of the ventricle adjacent to the caudal HF. I quantified three morphological types of microglia: ramified; which circulate the brain under normal conditions, rod microglia; which are a transitional morphology, and ameboid; which are activated by inflammatory cytokines during stress. Results showed more ameboid microglia than the other two morphologies measured across all hens in both walls of the ventricle, but there was no significant difference in microglia morphologies between minimal and severe KBF, while neuronal plasticity has previously been shown to be decreased in the latter. Furthermore, there was no correlation between the density of all three morphologies on the medial wall of the ventricle and DCX^+ density, which is a marker of neuronal plasticity in the caudal HF, or with KBF severity. These results suggest there were no morphological changes in microglia cells in the caudal HF between hens with minimal or severe KBF, indicating microglia do not change their morphology when fractures progress in severity.

4.2 Introduction

Microglia are a specialised population of macrophage-like cells and are often referred to as the resident immune cell of the brain. These cells are on constant patrol of the central nervous system (CNS) including the hippocampus and respond to pathogens and damage (Zhou et al., 2017; Zanto et al., 2011). Their activation is considered a hallmark of neuroinflammation leading to morphological changes depending on the microenvironment of the brain (Zhou et al., 2017). Under normal circumstances microglia scout the microenvironment in what has become known as their M2 phenotype which produce anti-inflammatory mediators such as interleukin (IL)-10, transforming growth factor (TGF)-β and brain derived neurotrophic factor

(BDNF) (Guo et al., 2022). When in their M2 phenotype, which is also known as the 'alternative activation', microglia work to maintain homeostasis by promoting phagocytosis of cell debris, tissue repair and supporting neuronal survival by secreting neurotrophic factors including BDNF (Pérez-Domínguez et al., 2017). Under M2 morphology, they appear as cells with many long branched processes (Vidal-Itriago et al., 2022) which is often known as a ramified morphological state. Upon neurological insult, microglia are quick to transition to their classical M1 state, where they begin to secrete inflammatory cytokines including IL-1β and IL-6 (Zhou et al., 2017). This change in morphology is often initiated by interferon-y (IFN-y) and lipopolysaccharide (LPS) (Colonna & Butovsky, 2017). M1 microglia have a different morphological shape to the M2 ramified type. M1 microglia retract their processes and have an enlarged cell body known as the ameboid morphological state. It is in this morphology microglia are considered to produce inflammatory reagents leading to neuronal damage through production of reactive oxygen species and inducible nitric oxide synthase (iNOS) leading to nitric oxide (NO) production (Jurga et al., 2020; Guo et al., 2022).

Not only do M1 ameboid microglia produce inflammatory cytokines, they have also been associated with adult hippocampal neurogenesis (AHN) in the mammalian brain. In the absence of neurogenic threats such as stress or pathogens, M2 ramified microglia are modulators of hippocampal neurogenesis through the pruning of newly born neurons (Gemma & Bachstetter, 2013). As new cells emerge, various other cells within the circuit become apoptotic which microglia are able to engulf following programmed cell death in the absence of inflammation and damage associated molecular patterns (DAMPs). Microglia are able to modify their processes when in ramified morphology to form phagocytotic pouches at the terminal branch of the process and engulf the apoptotic cell (Gemma & Bachstetter, 2013; Greene & Pisano, 2012b). On the other hand, in the presence of tissue damage, ameboid M1 microglia respond to damage associated molecular patterns DAMPs and following the secretion of inflammatory cytokines, they phagocytose neurons through their soma (Gemma & Bachstetter, 2013). When microglia are activated under their M1 ameboid phenotype, the release of inflammatory cytokines leads to expression of NO producing iNOS and major histocompatibility complex-2 (MHC-2) which contribute to neuronal damage by antigen presentation which can lead to apoptosis (Guo et al., 2022). When iNOS expression leads to an increase of NO, this can induce energy depletion-induced necrosis and tissue damage. One study in the avian species, which investigated iNOS expression in the quail retina, suggested that under normal circumstances LPS treatment induced an increase of ameboid microglia and upregulation of iNOS. However, that study did not investigate associated changes in neurogenesis (Sierra et al., 2014). A further study in Japanese Quail which looked at pre vs post-natal stress found expression of IL-1β and CSFR-1 was increased and IL-10 decreased in birds subjected to post-natal stress in the hippocampus and hypothalamus, but the morphological type of microglia remains unclear (Walker et al., 2019a). It is clear there is a link between inflammation producing M1 microglia and the decline of neurogenesis in the CNS in mammalian research and some emerging evidence in the Japanese Quail and Quail Retina models, however there is not sufficient evidence to suggest M1 ameboid microglia are involved with the decline in AHN found in the birds with severe keel bone fractures (KBF) discussed in the previous chapter and found by Armstrong et al 2020.

Microglia are not restricted to the ameboid M1/ ramified M2 morphologies: recently emerging evidence has supported a striking morphological type known as rod microglia. Rod microglia are visually different with the absence of primary and secondary processes and a narrowing of the cell and soma (Holloway et al., 2019). Little is known about rod microglia, however, they have been associated with ageing and neurodegenerative diseases. It remains unclear if rod microglia are present as a result of healthy ageing or age-related neurodegeneration (Holloway et al., 2019). Rod microglia have been observed in the hippocampus in models of epilepsy (Wyatt-Johnson et al., 2017; Greene & Pisano, 2012a). Epilepsy is known to provoke ramified microglia which then produce inflammatory cytokines including IL-1β and TNF-α (Wyatt-Johnson et al., 2017). That study found an increase of rod microglia two-weeks after induction of epilepsy in the adult rat pilocarpine model affecting the CA1 region wrapped along apical dendrites of pyramidal neurons (Wyatt-Johnson et al., 2017). It is not clear what the function of rod microglia may be, but one hypothesis is to prevent further damage at a site of injury (Au & Ma, 2017). There is little known about the presence of rod microglia in birds and these observations have mainly been made in mammalian research.

In the previous two chapters colony stimulating factor -1 (CSFR-1) was used as a genetic marker where if increased, this would indicate greater activation of microglia within a specific group. However, whilst CSFR-1 is a commonly used marker for microglia activation, it is expressed by microglia during all active morphological states and microglia in the adult mammalian brain are actually dependent on CSFR-1 signalling (Renee et al., 2015). This means the previous two chapters tell us little about the microglia behaviour and hippocampus environment. In addition, if the occurrence and severity of when a KBF occurred impacts gene expression of IL-6, IL-1β and IL-10, then it could be expected to see varying levels of microglia morphology throughout the birds used for this study depending on fracture status. In this chapter the hippocampus from the birds used in the previous chapter and by Armstrong et al 2020 with minimal or severe KBF will be stained using allograft inflammatory factor-1, also known as ionized calcium-binding adapter molecule 1 (IBA-1). Staining will be performed for the three morphologies: ameboid, ramified and rod. The lateral (nidopallial) and medial (hippocampal) wall of the lateral ventricle will be counted for IBA-1^+ microglia. IBA-1 is a calcium binding protein specifically expressed by macrophages and microglia once activated (Wittekindt et al., 2022). IBA-1 is commonly used to detect microglia in mammals and considered a sensitive method to detect changes in microglia morphology including processes to determine activation state (Wittekindt et al., 2022; Zhou et al., 2017). This antibody has been widely used in various mammalian and avian studies as a reliable marker of microglia (Viana et al., 2013; Diniz et al., 2016). The subventricular zone (SVZ) has been chosen as a counting zone for density as the SVZ is considered a neurogenic niche with microglia showing unique phenotypic features and are more active in this region according to mammalian research (Matarredona et al., 2018).

This main hypothesises for this chapter are that keel bone damage triggers inflammation and predicts that there could be a greater density of ameboid microglia as a consequence in the HF of birds with severe KBF. This is based on previous findings in chapter 3 which suggest newly acquired KBF have increased IL-1β expression, which is not present in older fractures and another study in hens where IL-1β and IL-6 expression was increased in birds with fractures. It could be possible that during the initial stage of a KBF, inflammatory cytokines are expressed which eventually leads to more ameboid microglia in the HF. This might also correlate with DCX^+ neuron density and the severity of KBF where IL-6 expression increased with greater severity. Together with the results from the previous chapter where cytokine expression was influenced by the age and severity of the fracture, increased ameboid microglia could point towards inflammation driven AHN reduction in birds with KBF.

4.3 Methods

4.3.1 Ethical Statement

This experiment was granted ethical approval by the Animal Welfare and Ethical Review Body at Newcastle University, project ID AWERB 549.

4.3.3 Animals and Housing

Alongside the previous chapter the animals used in this study were obtained as a follow up study from our lab's previous work on keel bone fractures (Armstrong et al., 2020; Rufener, Baur, et al., 2018). A summary of the animal conditions can be found in the previous chapter with a full explanation in our lab's previous publication (Armstrong et al., 2020).

4.3.4 Keel bone assessment/ fracture severity scoring

This method has been outlined in the previous chapter, with a full summary in our lab's previous paper and full details on fracture severity scoring in our lab's previous collaborations (Armstrong et al., 2020).

4.3.5 Tissue and processing

The samples collected for this chapter are outlined in the previous chapter and all samples were obtained by the author of our lab's previous work (Armstrong et al., 2020). For this study and work by Armstrong et al 2020, the hippocampus from the alternate hemisphere used in the previous chapter was immersion fixed in 4% paraformaldehyde in 0.1M Phosphate Buffered Saline (PFA-PBS) at 4^0C for 44-48 hours to prepare for immunohistochemistry analysis. As described in work by Armstrong et al 2020 which this work follows on from, and for the purpose of balancing possible lateralisation of AHN, tissue collection for each purpose was balanced between the left and right hemisphere within both KBF groups. Next, samples were cryoprotected in 30% sucrose in 0.1M PB solution to then be embedded in optimal cutting temperature embedding medium (OCT) (4583, Electron Microscopy Sciences – USA). Coronal sections of 50 μm were cut on a cryostat (HM 550, Microm – Germany) and stored in a cryoprotectant solution of 30% Glycerol, 30% ethylene glycol, 0.1M PBS at 20^0C. Serial sections taken at 400 μm interval were processed for immunohistochemistry.

4.3.6 Immunohistochemistry

To quantify the number of microglia and morphology types, sections were stained for IBA-1. Our primary antibody was rabbit anti-IBA-1 (product code 019-19741) (FUJIFILM Wako Chemicals) at a concentration of 1:500. Anti-IBA-1 which has mainly been used in the mammalian species was tested and developed in Chicken HF tissue for the purpose of this study, although this antibody has already been used in other avian studies including Sandpipers (Diniz et al., 2016). Testing was performed on HF tissue in a range of antibody dilutions between 1:250 and 1:2500 before confirming 1:500 to be the most appropriate volume of antibody. This was with secondary antibody biotinylated goat anti-rabbit IgG (Vector Labs, BA-1000), which was again tested at a range of concentrations. Development of this staining was also tested with different incubation temperatures including room temperature (17-20^{o}C) and at 4^{o}C in a cold room both overnight. Overnight at 4^{o}C was the preferred method.

Staining was performed across two batches, each balanced for minimal and severe KBF groups. Free-floating tissue slices were firstly washed in 0.1M PBS for three rounds of five minutes. Next, samples were incubated for 30 minutes for endogenous peroxidase inhibition in 1% H_2O_2 (Sigma Aldrich, UK). Samples were then washed again for 3x5 minutes in 0.1M PBS to then be incubated for 60 minutes in blocking solution consisting of 2% goat serum and 1% Bovine Serum Albumin (BSA) dissolved in 0.1M PBS with 0.3% Triton X-100. Distilled H_20 was used to quickly rinse samples before primary antibody incubation at 1:500 with rabbit anti-IBA-1 with 0.3% PBS triton X-100 overnight at 4^{0}C. Again, samples were washed 3x5 min in 0.1M PBS, then incubated at room temperature for 120 minutes in biotinylated goat anti-rabbit IgG secondary antibody at a concentration of 1:250 with 0.1% PBS triton X-100. PBS wash (3x5 minutes) was performed before addition of conjugate enzyme in 1:250 Horse Radish Streptavidin (Vector Labs, SA-5004) for 60 minutes. Samples were washed in 0.1M PBS (3x5) then dH_20 (1X5) before chromogen incubation for 30 seconds in 3,3'-Diaminobenzidine (DAB) diluted with SIGMAFAST tablets and ultrapure water at a concentration of 0.35 mg/ml. Samples were immediately rinsed in dH20 to abort the reaction then washed again in 0.1M PBS (3x5). Samples were then mounted on to gelatine-subbed sides with a paintbrush in dH_20. Once dry, nuclei were stained using a methyl green solution made of 0.5g methyl green (Sigma) and 100ml of 0.1M sodium acetate buffer at pH 4.2. The dried sections were soaked in dH_20 before addition of the methyl green solution for 5 minutes at room temperature, then transferred back to dH_20. A dehydration process was performed dipping the slides 10x each in 95%, 100% and a change of 100% alcohol. Then slides twice submerged in histoclear for 5 minutes before coverslip attachment using Eukitt (03989 FLUKA).

4.3.7 Quantification of Microglia

For each animal, 2 sections corresponding to the lateral ventricle wall and medial ventricle wall, best representative of Interaural 1.36 mm in the Chicken Brain Atlas (Puelles et al., 2013) were analysed. This enabled analysis of two counting frames for the medial (x=7, y=5.1) and lateral (y=7, y=5.3) ventricle wall of the caudal HF. In the mammalian literature microglia are considered a key component of the SVZ neurogenic niche alongside the hippocampus, where they are densely populated and active (Matarredona et al., 2018). Sections were analysed with an optical microscope (Lecia DMLB, Germany) composed with a digital video camera (Lecia DMLB, Germany), and motorized stage system (Lecia AGM, Germany). Quantification was performed blind to KBF status and by a single observer.

Image analysis was performed using Stereo Investigator software (version 2018.1.1, MBF Bioscience, USA). Both ventricles for counting were outlined at 2.5x magnification on all samples and the counting frame was measured in µm per region to account for discrepancies per sample/animal. Cell density was the counted at 100x magnification.

Cell density across the medial and lateral ventricle wall was split across three morphological states of microglia: i) ramified consisting of a smaller nucleus and multiple longer branching processes , ii) rod shaped containing one single process branching from the cellular body and nuclei and iii) ameboid which have few or completely retracted processes and a swollen cell body (Choi et al., 2022) (figure 3). Cell counts were transported to MS Excel to manually calculate the densities of all three morphologies. To calculate density, the length of the ventricle wall where microglia counts were performed was measured. For each morphological cell category, I combined the cell counts of both sections counted per bird for the lateral and medial wall of the ventricle separately. I also added up the lengths of the lateral walls from both sections, and the medial wall from both sections. The number of cells was then divided by the length of the relevant ventricle wall, which gave separate densities for all three morphologies on each wall of the ventricle.

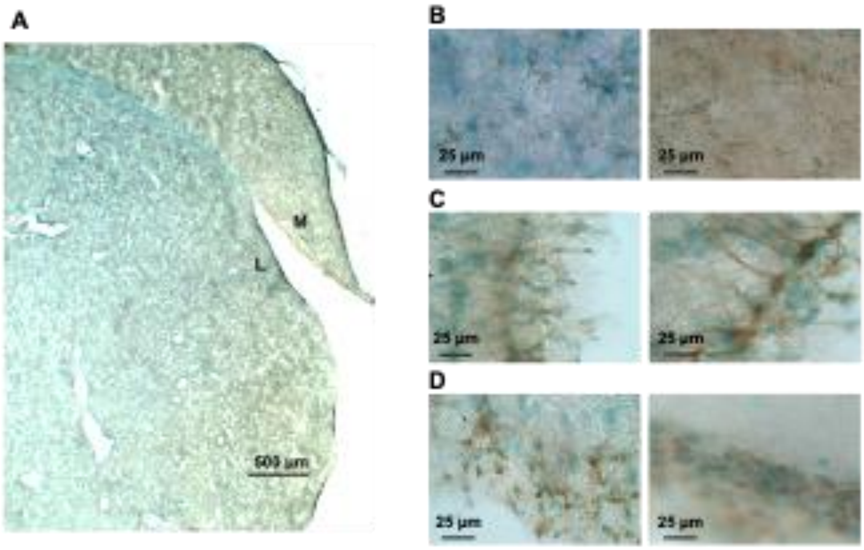

Figure 13: The three microglia morphologies: Ameboid, Rod and Ramified counted.

(A) the lateral (L) and medial (M) ventricle wall near the caudal HF at 2.5x magnification, (B) Ameboid (C) rod (D) ramified IBA-1+ stained microglia with methyl green nuclear staining at 100x magnification in the caudal HF of birds with minimal or severe. These images were obtained as part of my own work from the tissue analysed in this study.

4.3.8 Statistical analysis

Cell density was calculated using MS Excel where a Zi was calculated to normalise for variation across staining batch. The Zi was calculated from the formula $Z = \frac{x-\mu}{\sigma}$. Cell densities (Zi) were then exported to R Studio (version 1.3.959) where the linear mixed effects model (LMER) with type 3 ANOVA was performed using the AFEX package. Within this model the ventricle wall and microglia morphology were both within-subject factors, KBF severity group was a between-subject factor and bird ID was included as a random factor. Density (Zi) of all 3 morphological states was compared between KBF status of the bird, the medial or lateral ventricle. The Zi for multipolar and bipolar (DCX^+) density counts were provided as part of our lab’s previous study on KBF fractures (Armstrong et al., 2020) and used as part of a correlation analysis using the LMER and type 3 ANOVA with IBA-1^+ density (Zi). Multipolar and Bipolar DCX^+ density obtained from Armstrong et al 2020 were compared against IBA-1^+ density. Fracture severity or group were not included in this model, although bird ID was included as a random factor. Statistical significances of $p<0.05$ are listed in text where appropriate, full statistical analysis can be found throughout with reference to table. Graphs and figures were produced by using GGPLOT2 and GGPUBR packages in R studio.

4.4 Results

4.4.1 Density of IBA-1$^+$ Microglia

This chapter quantified the density of three distinct morphology types in birds with minimal and severe KBF groups. There was no statistical significance between KBF fracture group, suggesting no impact of fracture on IBA-1$^+$ cell density at the time these samples were obtained (table 15, figure 15). There was also no significance between the lateral or medial ventricle, suggesting no variation between each site. There was however a significant effect of cell type (F(2,90)=44.3713, P=3.896e-14), suggesting all birds had differences in the morphological states of microglia. Pair-wise analysis determined the ameboid morphology were statistically greater than ramified (t(98)= 5.526, P=0.0001) and rod (t(98)= 9.380, P=0.0001), however ramified and rod were also significantly different from one another (t(98)= 3.854, P=0.0006) (figure 15).

Table 15: Density of IBA-1+ microglia morphologies in the medial and lateral ventricle of the HF in birds with minimal or severe KBF

Output from the linear mixed model with three-way ANOVA of IBA-1$^+$ Density (Zi) of three morphological states in the medial and lateral ventricle of the caudal HF between Minimal and Severe KBF birds. All significant effects (p<0.05) are highlighted in bold.

Density of IBA-1$^+$ Microglia cells	Fracture Group	F(1,17)=0.5085, P=0.4853
	Morphology	**F(2,90)=44.3713, P=3.896e-14**
	Ventricle	F(1,90)=0.6217, P=0.4325
	Fracture Group : Morphology	F(2,90)=0.9588, P=0.3872
	Fracture Group : Ventricle	F(2,90)=0.9588, P=0.3872
	Morphology : Ventricle	F(2,90)=1.0909, P=0.3403
	Fracture Group : Morphology : Ventricle	F(2,90)=1.1795, P=0.3121

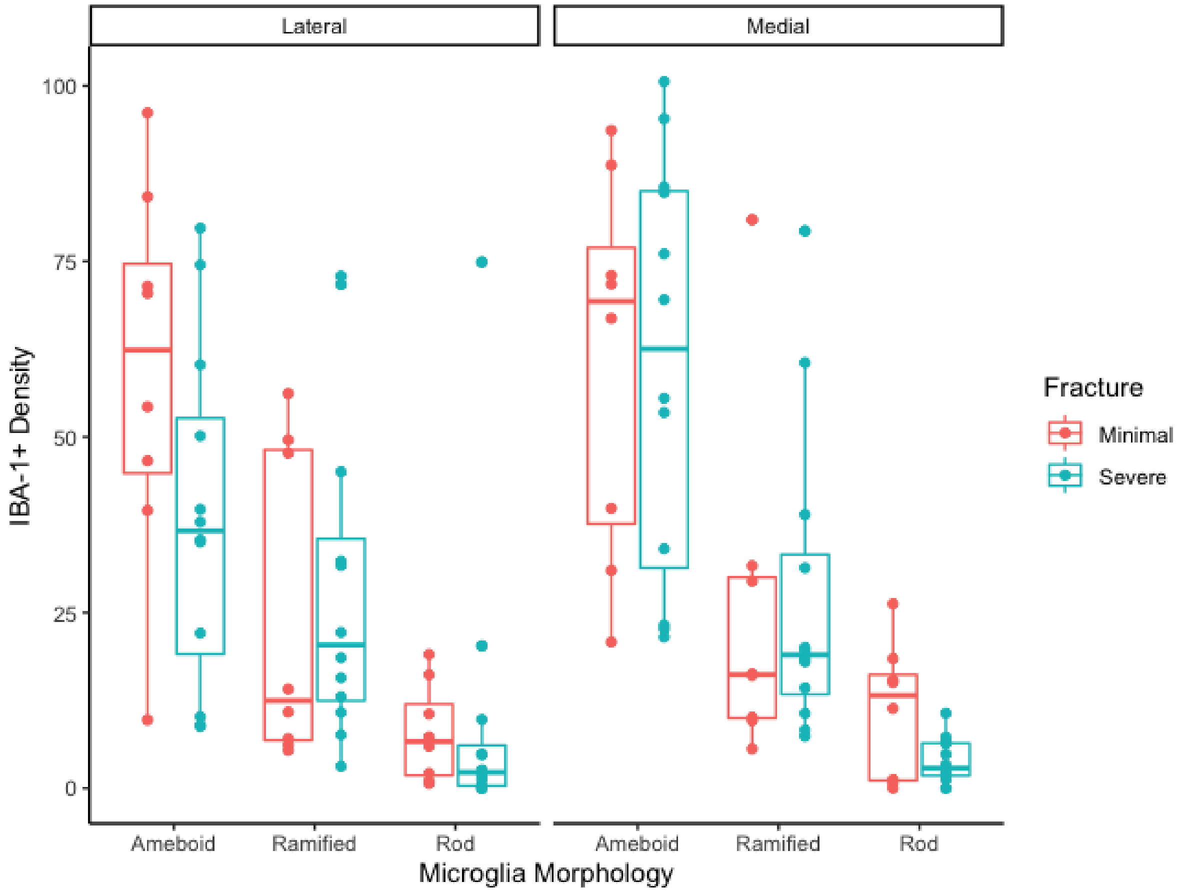

Figure 14: Density of IBA-1+ Microglia in the medial and lateral ventricle of birds with minimal and severe fractures

Density of IBA-1+ Microglia categorised into three morphology types: Ameboid, Ramified and Rod in the lateral and medial ventricle of the caudal HF in birds with minimal or severe KBF. The line visualises the median of each group with the hinges representing the upper and third quartile. Sample n= severe 24: medial 24 lateral, minimal: 24 medial 24 lateral.

4.4.2 Severity score

To determine if microglia morphology changes depending on the severity of the KBF, I compared all densities with the actual severity score of each KBF from all individual birds in place of the group's minimal vs severe. Again, there were no statistically significant differences between IBA-1^+ Microglia density across all morphologies and the KBF severity score, across both sites counted (table 16).

Table 16: Correlation between the density of IBA-1+ morphologies across the lateral and medial ventricle of the HF and the KBF severity score

Output from the linear mixed model with three-way ANOVA of IBA-1$^+$ Density (Zi) of three morphological states in the medial and lateral ventricle of the caudal HF, correlated with the individual KBF severity score of the corresponding bird.

Density of IBA-1$^+$ Microglia	**Morphology**	**F(2,90)=44.3713, P=3.896e-14**
	Ventricle	F(1,90)=0.6217, P=0.4325
	Severity score	F(1,17)=1.1869, P=0.290636
	Severity score : Morphology	F(2,90)=0.3368, P=0.714925
	Severity score : Ventricle	F(1,90)=0.1178, P=0.732230
	Morphology: Ventricle	F(2,90)=0.2602, P=0.771487
	Severity score : Morphology : Ventricle	F(2,90)=1.1544, P=0.319883

4.4.3 Time point of first and last fractures

To address the possibility of differences between new and old fractures and microglia, I compared microglia density in the same model with the time point of the first recorded fracture and then the last recorded fracture. There was no statistical significance between all microglia morphologies, ventricle and the time point of the first (table 17) and last (table 18) recorded severity score. Thus, potentially ruling out the possibility of older fractures (present at an earlier time point) and newer fractures (present at a later time point) correlating with IBA-1$^+$ density.

Table 17: Correlation between the density of IBA-1+ morphologies across the lateral and medial ventricle of the HF and the first observation of a KBF

Output from the linear mixed model with three-way ANOVA of IBA-1$^+$ Density (Zi) of three morphological states in the medial and lateral ventricle of the caudal HF, correlated with the time point of the first reported KBF for the corresponding bird.

Density of IBA-1$^+$ Microglia	**Morphology**	**F(2,90)=44.3713, P=3.896e-14**
	Ventricle	F(1,90)=0.6217, P=0.4325
	First score	F(1,18)=0.4865, P=0.49441
	First score: Morphology	F(2,90)=0.4018, P= 0.67031
	First score : Ventricle	F(1,90)=0.5861, P=0.44592
	Morphology : Ventricle	F(2,90)=0.7838, P=0.45976
	First score : Morphology : Ventricle	F(2,90)=0.2489, P=0.78018

Table 18: Correlation between the density of IBA-1$^+$ morphologies across the lateral and medial ventricle of the HF and the last observation of a KBF

Output from the linear mixed model with three-way ANOVA of IBA-1$^+$ Density (Zi) of three morphological states in the medial and lateral ventricle of the caudal HF, correlated with the time point of the final recorded KBF (+1 on the severity scoring scale from the prior score) of the corresponding bird.

Density of IBA-1$^+$ Microglia	**Morphology**	**F(2,90)=44.3713, P=3.896e-14**
	Ventricle	F(1,90)=0.6217, P=0.4325
	Last score	F(1,18)=0.2978, P=0.5919
	Last score: Morphology	F(2,90)=0.7815, P=0.4608
	Last score : Ventricle	F(1,90)=0.3151, P=0.5760
	Morphology : Ventricle	F(2,90)=0.6273, P=0.5363
	Last score : Morphology : Ventricle	F(2,90)=0.2175, P=0.8049

4.4.4 Density of IBA-1$^+$ Microglia and DCX$^+$ Multipolar and Bipolar Neurons

To determine any potential relationship between microglia and neuron density, a correlation analysis was performed between IBA-1$^+$ density on the medial ventricle wall and DCX$^+$ density (Zi) of the caudal HF. It could be determined from table 5 that there is no correlation between the density of IBA-1$^+$ microglia between all three morphologies and medial ventricle, and the density of multipolar or bipolar DCX$^+$ neurons across the caudal HF. Therefore, in this case microglia density does not predict multipolar or bipolar neuron density in the caudal HF.

Table 19: Correlation between IBA-1$^+$ morphologies and DCX+ density in the caudal HF

Output from the linear mixed model with three-way ANOVA to determine a statistical correlation between IBA-1$^+$ Microglia density (Zi) across the medial ventricle and three morphology states and density of DCX+ Bipolar and Multipolar neuron density (Zi) in the caudal HF. Multipolar and Bipolar density were ran as two separate covariates.

Density of IBA-1$^+$ Microglia cells	**Morphology**	**F(2,90)= 44.3713, P=3.896e-14**
	Morphology: Multipolar Caudal Multipolar Caudal	F(2,51)= 0.1494, P=0.8616 F(1,51)= 0.8109, P=0.3721
	Morphology: Bipolar Caudal Bipolar Caudal	F(2,51)= 0.2886, P=0.7506 (F1,51)= 0.0065, P=0.9363

4.5 Discussion

This chapter used IBA-1 immunohistochemistry to measure the density of three morphological types of microglia in the medial and lateral ventricle wall of the telencephalon, in birds with severe or minimal KBF. This study predicted the birds with severe fractures would have increased ameboid microglia density which could indicate a role for inflammation driven M2/ameboid microglia in the HF of birds with severe KBF, which was not the case.

Results show there were no significant differences across all morphologies between birds with severe vs minimal KBF, nor were there any differences in microglia between the medial and lateral ventricle walls. There were however significantly more ameboid microglia than rod and ramified present overall, which did not change amongst fracture status or ventricle wall (figure 14, figure 15). As the samples available for this study were all obtained on one occasion and did not include any birds with absent fractures, it can be speculative that more ameboid microglia are a consequence of KBF. Although, this could be possible. As the findings of this study are limited due to the absence of birds without a KBF, a correlation analysis between the time the fracture occurred and the severity score for each bird was run with microglia density. It could be hypothesised that in birds whose fractures were acquired closer to the time these samples were obtained, representing newer fractures, microglia morphological types could differ compared to older fractures. Results reveal there was no correlation between the severity score and microglia density of all morphologies in both ventricles, neither was there any correlation between the time the fracture occurred. These results suggest there is no relationship between the severity or the age of the fracture and all three microglia morphology types. To this date little research has measured different morphological types of microglia in birds which is a benefit of this study. This result is somewhat surprising as in the previous chapter, IL-6 increases with fracture severity and IL-1β is increased whilst IL-10 decreases in birds who had fractures detected earlier in the caudal HF with the reverse effect in the rostral and ameboid microglia produce these cytokines (Reemst et al., 2016). As mentioned, the limitations of this study meant it was not possible to include birds without KBF. Therefore, the birds were categorised into minimal (severity score under 5) and severe (severity score over 5). This means that whilst severity of the fracture in this study does not appear to be related to the presence of ameboid microglia, the presence of a fracture could be. For future work, it would be of interest to see if there would be differences in ameboid microglia between birds with and without fractures.

KBF in hens is already known to lead to the production of inflammatory cytokines in the whole brain as (Wei et al., 2019) found KBF leads to the onset of stress and increased IL-1β, TNF-α and iNOS. However, this was not specific to the hippocampus where (Armstrong et al., 2020) found a decrease in DCX^+ neuron density in birds with severe fractures. It is likely that cytokines are being produced at the fracture site rather than the hippocampus, for example cytokines are involved with the recruitment of inflammatory cells including T and B cells (Bahney et al., 2019). This study and the study in chapter 4 did not investigate the expression of cytokines in anywhere other than the hippocampus, therefore it is of interest to explore the possibility of measuring expression in the blood or spleen during the early stages of a fracture. Armstrong and colleagues (2020) found multipolar and bipolar neuron density were lower in birds who acquired fractures earlier with a longer duration in the caudal HF, but not rostral suggesting the caudal HF is more sensitive. This same study also found that the time point when a hen first acquired a KBF negatively correlated with their fracture severity at the final scoring time point but did conclude the final time point was the best predictor of DCX^+ density. This could mean categorising birds into minimal vs severe fractures is not the most optimal way of determining the potential role of microglia in mediating the reduction of AHN. As whilst DCX^+ density is sensitive to fracture status, ameboid microglia appear to increase in density irrespective of the fracture severity or when it occurred. It remains unclear whether it is a generally higher number of ameboid microglia compared to ramified and rod, or if this is an increase due to the presence of the fracture. There was also no relationship between any microglia morphology and multipolar or bipolar density in the caudal and rostral HF suggesting this could be a general increase rather than a contributor to decreased AHN. In addition, due to tissue availability this study only measured density in the caudal HF. It may be a possibility that there is a greater presence of ameboid microglia in the medial and lateral ventricle of the caudal HF in comparison to the rostral which appears to be less sensitive to KBF induced AHN suppression (Armstrong et al., 2020).

Although in this study it appears there is no relationship between the morphology of microglia in birds with severe vs minimal KBF, neuroinflammation is not limited to the presence of a specific subtype of microglia cell. Ameboid microglia are known to secrete iNOS, an enzyme which leads to the production of iNOS-derived NO during stress (Zhu et al., 2014). iNOS expression has been shown to contribute to the pathological development of cerebral ischemia. When $iNOS^+$ cells were depleted in mice there was suppression of NO and the inflammatory response which collectively led to the prevention of neuronal damage (T. Li et al., 2022). Alongside iNOS, microglia produce a central element of the complement cascade known as C3. A study using early-stage experimental autoimmune encephalomyelitis (EAE) in female mice showed upregulation of C3 in the dentate gyrus (Bourel et al., 2021). When this study inhibited C3 they observed preservation of memory performances and reduction in microglial synaptic loss in the dentate gyrus of the mice in this study, both of which are characteristics of multiple sclerosis (MS) present from early stages of disease onset. Increased iNOS was observed in birds with KBF by (Wei et al., 2019) and C3 is produced by osteoclasts to aid fracture healing (Ehrnthaller et al., 2013). So measuring the expression of these markers could be useful to determine any involvement within the HF of birds with KBF.

Not only do microglia secrete other markers including iNOS and C3 in response to brain injury that this chapter has not yet explored, they also interact with astrocytes in depression-like mice (S. Li et al., 2022). Astrocytes are another common glial cell in the CNS which regulate brain functions and have been associated with neurodegenerative conditions (S. Li et al., 2022). Astrocytes are thought to have similar morphological changes to microglia including the A1 astrocyte which can cause neuronal death and the A2 astrocyte which has a neuroprotective role similar to the M2 microglia (S. Li et al., 2022; Biscaro et al., 2012). S. Li et al 2022 who induced mild chronic depression in mice demonstrate the involvement of A1 astrocytes, which was accompanied by dendritic atrophy of hippocampal neurons. This study also showed evidence of the microglial NLRP3 inflammasome which activates caspase-1 leading to production of IL-1β and various other inflammatory cytokines. Primary astrocytes isolated from NLPR3$^{-/-}$ mice were treated with LPS-activated microglial-conditioned media and they found an increase in A1 astrocyte markers. This study also demonstrates the involvement of A1 astrocytes in mediating neuronal death through testing various A1 markers including C3 which were all elevated in the hippocampus of the chronic stress mice at 4 weeks into stress. At this time there was no onset of depression-like behaviour and neuron marker NeuN was not reduced in the hippocampus, but postsynaptic density protein-95 (PSD-95) which is an important postsynaptic scaffolding protein in synaptic plasticity showed lower intensity during fluorescent staining. This could indicate loss of synaptic plasticity. The results from S. Li et al 2022 taken together with the results from this chapter showing increased ameboid microglia in all birds and increased IL-1β in birds with KBF (Wei et al., 2019) and reduced AHN and a depressive-like state in birds with severe KBF (Armstrong et al., 2020) do create opportunities for future work that has not been covered in this chapter. This study demonstrates increased ameboid microglia in the hippocampus of birds with KBF and in the previous chapter increased IL-6 as the severity of the fracture increased and increased IL-1β decreased IL-10 when birds acquired fractures earlier. This does open the possibility for exploring the involvement of further astrocyte and microglia markers including C3, iNOS, and NLPR3 in mediating the stress-induced decrease in AHN found in previous hen studies (Armstrong et al., 2022; Gualtieri et al., 2019; Armstrong et al., 2020). The complement system in chickens is thought to be highly similar to mammals , for example complement component-4 which is one of the MHC genes, is involved with innate immunity in chickens similarly to mammals (Garcia et al., 2021; Kaufman, 2022). Although C3 has been involved with neurotoxic astrocytes in human models (S. Li et al., 2022), this is yet to be explored in chickens. However as this and the previous chapter did not include any birds with absent fractures, a new method of ensuring stress and accurate controls such as unpredictable chronic mild stress (UCMS) might be more beneficial.

4.6 Conclusion

To conclude, this chapter used IBA-1 to measure cell density of ramified, ameboid and rod microglia in the medial and lateral hippocampus of birds with minimal and severe KBF. This chapter found no differences in microglia morphologies across the medial and lateral ventricle walls and no differences between minimal and severe fractures. There was a higher density of ameboid microglia than the other counted morphologies across all birds, suggesting greater presence of inflammatory M1 microglia. Although the results from this study are limited as there could be no comparisons made between birds with a fracture vs no fracture. It remains unclear if there is a general rise in ameboid microglia versus ramified and rod, of if this due to fracture irrespective of the severity. Microglia express inflammatory markers which have not yet been explored in the hippocampus of hens with reduced AHN including iNOS, C3 and NLPR3 and have been shown in mice to upregulate NLPR3 leading to the induction of neurotoxic astrocytes. For future studies it would be interesting to explore these markers to determine the involvement of mediating the decrease in neuron density in the hippocampus of stressed hens.

CHAPTER 5: DOES THE EXPRESSION OF INFLAMMATORY CYTOKINES AND INFLAMMATORY MARKERS OF ASTROCYTES CHANGE THROUGHOUT UNPREDICTIBLE CHRONIC MILD STRESS?

5.1 Abstract

Unpredictable chronic mild stress (UCMS) was recently shown to reduce neuronal plasticity in the hippocampal formation (HF) of hens. Chronic stress is a great concern for the welfare of hens and interventions to combat this are utilised. This includes the early-life intervention of raising with a dark brooder (DB) which provides light and warmth as the bird hatches and is considered to reduce fearfulness. As chronic stress leads to reduced neuronal plasticity in the HF and has been associated with poor mood, the involvement of the immune system as a mediator is of interest to this study. Earlier findings in chapters 3 and 4 which explored expression of inflammatory cytokines in the hippocampus of birds with KBF and reduced neuronal plasticity, suggest increased IL-1β expression when a fracture first occurs and more ameboid microglia when a fracture of all severities is present. However, those chapters gave little opportunity to explore inflammation at the early stages of a fracture. This study uses UCMS in birds reared with DB and conventionally reared without DB, to determine if inflammatory cytokines are increased in the blood as UCMS progresses. In addition, this study is interested in the role astrocytes play in mediating the effects of chronic stress on neuronal plasticity in the HF by measuring astrocyte marker GFAP and markers of the inflammasome including caspase-1 (CASP-1), inducible nitric oxide synthesis-3 (NLRP3), complement protein-3 and nitric oxide producer iNOS. The expression of inflammatory cytokines IL-1β, IL-6 and TNF-α did not differ between UCMS and control birds, neither did expression differ between rearing groups suggesting stress or DB rearing does not increase inflammatory cytokine expression in the blood. These cytokines did increase in all groups from weeks 16 to 25, which could be due to the ageing of the bird rather than stress or rearing. There was however an increase of GFAP in the caudal HF of birds with UCMS, suggesting UCMS causes increased astrocytes in the caudal HF. There was also an increase in C3 gene expression in birds with UCMS compared to controls, although this increase was only observed in the birds with no DB. C3 levels were comparable in all DB birds to levels

in the conventionally reared UCMS group, suggesting C3 expression in DB birds is generally higher and not influenced by stress. Together, these results suggest UCMS could lead to increased astrocyte-mediated complement pathway activation in birds without DB rearing. Activation of the complement pathway could lead to phagocytosis of neurons within the HF and is of interest to future work.

5.2 Introduction

Unpredictable chronic mild stress (UCMS), which is an experimental model to induce chronic stress, is generally accepted to lead to a cascade of neurobiological effects. This includes decreased neuronal plasticity in the hippocampus and hypothalamus-pituitary-adrenal axis (HPA axis) dysregulation in adult birds (Gualtieri et al., 2019) and rodents, along with increased microglia cell activation and decreased brain-derived neurotrophic factor in rodents (Planchez et al., 2019). UCMS has been used amongst the mammalian and avian species as a controlled way to manipulate chronic stress over a fixed period of time, using stressors such as feed restriction at random intervals (Gualtieri et al., 2019; Robertson et al., 2017; Troubat et al., 2021). UCMS as a stressor hasn't been widely used in the Avian species, although it was previously found to suppress the incorporation of doublecortin-positive (DCX^+) neurons in the caudal pole of the hippocampal formation (HF). This was in hens receiving UCMS for 8 weeks compared to control hens who received no stress (Gualtieri et al., 2019), demonstrating chronic stress leads to reduced neuronal plasticity in a similar way to mammals. This chapter will build upon those previous findings by Gualteiri et al 2019 to explore the potential involvement of inflammatory and astrocyte-related markers. The early life of the hen is an important time and often shapes the development of characteristics including stress resilience and responsiveness later in life. Hens are typically reared in conventional commercial systems where their environment can have lasting effects as they age. For example, when hens are housed in cages they are considered more fearful than hens with enriched housing (Dumontier et al., 2022). During a young age, these hens are also considered to lack certain maternal characteristics provided by the maternal figure, including warmth. The Dark Brooder (DB) rearing is an intervention commonly used to support hens during early life to prevent fear and feather pecking. The rationale behind DB rearing is to provide maternal care in the absence of their broody mother hen who is not present for economical and disease prevention reasons (Nordquist et al., 2020).

When hens are raised without a maternal presence, DB rearing is thought to be a preferential rearing method to some as the inclusion of DB provides a dark space and warmth which would not be present in a conventional barn, which is required to maintain body temperature shortly after hatching. DB hens are thought to have several welfare and commercial benefits including reduced mortality caused by lower risk of cannibalism in adulthood, increased feather coverage and improved egg production (Nordquist et al., 2020). DB rearing is thought to reduce early life stress by providing welfare-enhancing care including shelter and separation of active and inactive birds. This was demonstrated in one study by testing duration of tonic immobility (TI) which was shorter in DB birds at all ages tested (Riber & Guzman, 2016). TI is a test used to test a fear-potentiated response in hens by removing the bird from their pen and placing one hand over the chest and another over the neck for 15 seconds. This particular study determined if the hen was motionless for 10 seconds after, the hen had entered TI and repeated if the hen responded earlier (Riber & Guzman, 2016). It was concluded the DB hens responded to this test earlier, alongside reduced feather pecking suggesting this method of brooding reduces fear and stress (Riber & Guzman, 2016).

With that in mind, it is not yet known if hens reared as DB are any less susceptible to the effects of chronic stress later in life in comparison to those reared in conventional housing, or if either produces an inflammatory response. Therefore, this study will use UCMS as a controlled method of inducing chronic stress, with an appropriate control group in hens with and without DB rearing. It is of interest to this study if hens subject to UCMS treatment produce increased inflammation-related markers based off if they were reared conventionally or as DB.

It has recently been demonstrated in the mammalian literature the involvement of inflammation during chronic stress in the dentate gyrus (DG) of the hippocampus leads to changes, such as increased interleukin-1β (IL-1β) secreted by microglia which has not yet been shown in birds. One study which recently used UCMS in P2X7-receptor knockout mice (P2XRR KO) found mice lacking this receptor exhibit decreased ventral AHN and less recruitment of microglia cells (Troubat et al., 2021). The P2X4R receptor is an adenosine triphosphate ligand-gated cationic channel receptor. It is expressed by microglia cells and has been shown to regulate inflammation (Fan et al., 2020). This is through the loss of intracellular potassium leading to activation of NLRP3 and proteolytic cleavage of pro-caspase-1 into its active form caspase-1 (Mountziaris & Mikos, 2008). In addition, P2X7R is also present on astrocytes which communicate with microglia (S. Li et al., 2022). Astrocytes have an important role throughout the brain, which includes the phagocytosis of synapses and modulate the immune response as antigen presentation cells (Pérez-Domínguez et al., 2017), which has not yet been explored in hens experiencing chronic stress.

This receptor, present on microglia and astrocytes, modulates potassium signalling leading to the release of inflammatory cytokine IL-1β which in turn stimulates the release of IL-6 and TNF-α, confirmed by P2X7R KO mice stimulated with lipopolysaccharide (LPS) (Troubat et al., 2021). Genetic depletion of P2X7R has also been shown to prevent neurogenesis alterations induced by chronic stress and can activate the nuclear factor (NF)-κB pathway to regulate AHN and impair generation of neurogenesis by inflammasome dependent IL-1β release (Koo et al., 2010).

Microglia are considered an essential component of the inflammatory process in the brain and are known to induce cytokine expression. However, inflammation in the brain often comes as a result of peripheral immune cells which communicate with astrocytes (Troubat et al., 2021). In a social defeat mice model, higher plasma levels of IL-1β, IL-6 and TNF-α has been correlated with greater depressive symptomology (Hassamal, 2023), which has not yet been explored in hens facing chronic stress. These cytokines typically do not cross the blood brain barrier (BBB) under normal circumstances, however during chronic stress the BBB is thought to become increasingly permeable to cytokines and nitro-oxidative stress molecules NO (Hassamal, 2023). One study in rodents subject to repeated water avoidance stress (WA) over a period of 10 days measured plasma protein expression of IL-1β, IL-6 and TNF-α and discovered IL-1β expression increases in a time dependent manner following WA exposure (Glovatchcka et al., 2008). This was matched with altered expression in the spinal cord of the astrocyte marker glial fibrillary acidic protein (GFAP) which was increased significantly at 24 hours past the fifth WA but significantly decreased at 24 hours past the tenth consecutive day of WA. Therefore, as in previous chapters there was no increase in the cytokines measured between the birds with chronic stress where DCX$^+$ density was decreased and the control group, it is of interest to this study if an increase in cytokine expression occurs during the early stages of UCMS and how long this increase is present. Astrocytes are essential for the maintenance of the BBB and can become compromised through retraction of their processes leading to increased penetration of peripheral substances entering the brain such as cytokines (Hodes et al., 2015). Alongside maintaining the BBB, under pathological circumstances astrocytes can cause neuronal death. A study which used chronic stressed mice demonstrated a neurotoxic A1 astrocyte (GFAP$^+$C3$^+$) was induced two weeks into the study prior to dendritic atrophy and behavioural impairments which came later at 6 weeks and remained present throughout the remainder of the study (S. Li et al., 2022). Complement protein-3 (C3) expression is a well-recognised characteristic of A1 astrocytes and expression was higher in depressed mice compared to control mice throughout the entire study, alongside GFAP in the hippocampus (S. Li et al., 2022). Increased C3 expression has also been associated with chronic stress in the prefrontal cortex of rodents and its function is said to activate of the complement pathway and tags synapses for removal by microglia (Pillai, 2022).

There is clear involvement of the immune system in rodent models of stress (Cain & Cidlowski, 2017; Gong et al., 2019) with reduced AHN. This is yet to be established in birds and the previous chapters have not established a clear link. Despite previous chapters measuring expression in the hippocampus of IL-1β, IL-6, TNF-α and BDNF in birds who had reduced AHN as a result of poor body condition and keel bone fractures (KBF), there was no clear correlation between doublecortin (DCX$^+$) density and cytokine expression. Neither was there any changes in cytokine expression amongst groups. Therefore, this chapter will use a UCMS protocol to induce chronic stress alongside control birds to further investigate the involvement of inflammation in birds which have previously been known to have decreased AHN in the caudal HF (Gualtieri et al., 2019).

The previous chapters have explored cytokine expression in the hippocampus of hens with varying physical conditions and keel bone fractures (KBF). However, there has been little evidence of IL-1β, IL-6 and TNF-α upregulation in those hens which had reduced AHN in the studies the previous two chapters were based on (Armstrong et al., 2022, 2020). In addition, there was no difference in the density of IBA-1$^+$ microglia morphological subtypes: ameboid, rod and ramified, in birds with minimal vs severe KBF. The previous two chapters had limitations including the absence of birds with no KBF and the selection of birds with varying physical conditions from a commercial farm.

Therefore, this chapter uses a different treatment to induce chronic stress. In this chapter UCMS was used as a controlled method to induce chronic stress over a set period of time. This also includes hens who received dark brooder (DB) rearing as chicks up to 4 weeks and those from conventionally reared systems. These hens received provisions including heat and a dark resting area. This chapter comes from a 2x2 study as part of a wider collaboration in our lab for Matt Craven's PhD project including 4 treatment groups: DB control, DB UCMS, conventionally-reared UCMS, conventionally-reared Control. To establish if inflammatory cytokines are increased in the periphery of hens subject to chronic stress, all cytokines measured in previous chapters will be repeated in the blood obtained across three separate time points using UCMS. This chapter provides a new opportunity to control the onset and offset of chronic stress. Therefore, it can be determined if cytokine expression is consistent across the UCMS period or if there is a gradual increase similar to rodents demonstrated by (Glovatchcka et al., 2008). In addition, this chapter will explore expression of nitric oxide (NO) producer iNOS in the hippocampus which is secreted by ameboid microglia in response to stress conditions including chronic exercise in rodents (T. Li et al., 2022). Finally this chapter will for the first time investigate the potential involvement of A1 astrocytes in the hippocampus by measuring GFAP which is present on many glial cells including astrocytes (Lambert et al., 2000). Other markers related with inflammation and cytokine secretion will be measured including C3 which activates the complement system and contributes to cell lysis (Boes, 2000). Alongside NLRP3 which activates caspase-1 (CASP-1) and triggers the secretion of inflammatory cytokines and CASP-1 which stimulates inflammatory induced cell death (S. Li et al., 2022). These will be measured in addition to a repeat of microglia activation marker CSFR-1 and predicts these markers will be increased in hens with UCMS.

The main hypothesis for this chapter is that there will be changes in the expression of inflammatory cytokines obtained from blood samples across three different time points of UCMS. This chapter predicts in conventionally reared hens, expression will increase mid-way through the UCMS protocol in comparison to before UCMS began and the control group. Expression will then decrease by the final time point when UCMS ends in line with the control group. This is when hippocampal tissue will be analysed, which this study predicts there will be an increase in the expression of astrocyte related markers. This study also predicts that there will not be the same increase in birds reared as DB's, where expression of the genes measured will not differ between control and UCMS birds which could indicate resilience towards UCMS.

5.3 Methods

5.3.1 Ethical Statement

This study was conducted under a Home Office licence PPL number PP7720523 for regulated procedures including UCMS and blood sampling. Post-mortem tissue collection was conducted under AWERB project ID number 549.

5.3.2 Animals and housing

The birds used in this study were obtained as part of a larger project within our lab for Matt Craven's PhD project.

Table 20: Timeline of events between weeks 12-25 of age. UCMS= unpredictable chronic mild stress

Age (weeks)	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Tonic immobility		X		X								X		
Pre-treatment	X	X	X	X	X									
Blood sampling					X				X				X	
UCMS						X	X	X	X	X	X	X	X	
Tissue Collection														X

A total of 48 H&N Brown Nick birds, split across 4 groups were used for this study. Treatment groups consisted of n=12 conventional with UCMS, n=12 conventional no stress, n=12 dark brooder UCMS and n=12 dark brooder no stress. Conventionally reared and DB-reared birds were reared with or without a dark brooder from ages 0-4 weeks. These were split across 4 rooms (w=2m x L=3.5m) each containing 12 birds (6 Conventional, 6 Dark Brooder). Birds were assigned to a room depending on results of a novel object test and tonic immobility, with an attempt to equalise the average TI result across rooms. This included placing 4 birds with the highest TI score into separate rooms to avoid placing all fearful birds into a single condition. Each room consisted of two pens, front and rear. The front pen contained nest boxes, perches, two feeders and water. The rear pen was empty. All rooms received minimal enrichment which was consistent between control and UCMS birds. This included nesting boxes, perches, grit on the floor, cardboard strips and tubes and

CD's. Birds also had access to ad libitum feed and water unless the stressor was "remove food/water" on that specific day. Birds were subject to several pre-treatment checks including tonic immobility and blood sampling, a timeline of this can be found in table 20.

5.3.3 Unpredictable Chronic Mild Stress (UCMS)

Rooms 1 and 3 received UCMS treatment for 8 consecutive weeks, rooms 2 and 4 were control groups. Birds subject to UCMS received various stressors outlined in table 21.

Table 21: Stressors used as part of the UCMS protocol with descriptions

Stressor	Description
Increased temperature	Temperature increased to 30ºC for 3 hours to be returned to baseline, unless signs of overheating were present in upwards of 50% of birds (panting, holding wings open and lethargy).
Long days	20 hours of light within a 24 hour period
Short days	4 hours of light within a 24 hour period
Wet bedding	Shavings were saturated with water using a hose from 3:30 onwards and replaced with dry shavings the next morning
Packed in boxes	All 12 birds within each room were packed into a red crate for 3 hours at any time of the day
Isolation	Each bird was placed into its own individual crate and kept as far apart from other birds as logistically possible
Chasing	A single human chased all birds for a maximum of 5 minutes up to 4 times per day with no less than 5 minutes between
Removal of food and water	Food and water was removed at 9:30am and returned approximately 1:30pm for water and 3:30pm for food. If signs of lethargy, panting or gathering where food/water would be placed for more than 30 minutes by upwards of 50% of birds, food and water was returned

5.3.4 Tonic immobility

All birds were placed on their back in a cradle with wing support and restrained with one hand on the bird's breast and eyes covered until the bird ceases to struggle. Tonic immobility (TI) was considered successful in this study if the bird was to remain still for 10 seconds after hands were removed from the bird's chest and eyes and repeated if not. If unsuccessful, TI was repeated for up to 4 times before a TI duration score of 0 seconds was given. Because TI was used to allocate birds within this study to groups, this data will not be analysed as part of this study. However, the TI analysis was utilised by Craven (2024).

5.3.5 Blood sampling

For the purpose of this study, blood was obtained on three occasions: pre-treatment at 16 weeks, mid-UCMS at 20 weeks and finally at the end of the UCMS protocol at 25 weeks. 250μl of whole blood was collected in 750μl TRIzol LS from the leg or brachial vein if the leg was not sufficient, protocol obtained from (Emami et al., 2021). Blood was collected after the bird spent 30 minutes in a bag as part of an acute stress manipulation.

5.3.6 Tissue collection

At the end of the UCMS period, all birds were killed by intravenous injection with pentobarbital and dissected over 4 days (n=12 per day). The brain was dissected from the skull, the HF was dissected from the brain and split into rostral and caudal portions to then be transferred to RNAlater tubes in the fridge for 48hours before being transferred into the freezer at -80^{0}C.

The spleen from all birds were also obtained and weighed, then a sample stored in RNAlater tissue storage reagent (Sigma-Aldrich, UK) for 48 hours at 4^{o}C, then frozen at -80^{o}C.

5.3.7 RNA extraction

Extraction of RNA was split across sample type, blood and HF. For the extraction of RNA from rostral and caudal HF samples, these have been described in chapter 2 section 2.3.4. For the extraction of RNA from blood samples, each extraction run included an equal proportion of blood from time points A, B and C. For each sample 200μl of blood was collected under the method described in section 5.3.5 then mixed with 800μl TriSure reagent (Bioline, London UK). This was then lysed in the Qiagen Tissue Lyser II (Qiagen LTD, Crawley UK). 200μl Chloroform was added to the homogenised sample before centrifugation at 13,000 RPM for 15 minutes at 4^{o}C. The aqueous phase was combined with an equal volume of 100% ethanol for RNA extraction using the direct-zol RNA miniprep kit (Zymo Research Cambridge Biosciences, UK). Alongside the rostral and caudal HF samples 1μg RNA was reverse transcribed using the SensiFast cDNA synthesis kit (Bioline, London UK) ready for RT-PCR.

5.3.8 RT-PCR

For the purpose of this study primers were designed using the NCBI primer-BLAST tool (National Centre for Biotechnology Information) or obtained from the scientific literature if stated. For all genes of interest, lamin-B receptor (LBR) was used as a reference gene for both HF and blood samples.

For analysis of blood, cytokines IL-6, IL-1β, IL-10, TNF-α and TGF-β were measured, full sequences can be found in table 1 of chapter 2, section 2.3.4. For rostral and caudal HF samples CSFR-1 (table 1, chapter 2), iNOS (Burggraaf et al., 2011), CASP-1 (S. Li et al., 2022)and NLRP3 (Karaffová et al., 2020) complement protein-3 (C3) (Zhou et al., 2023) and GFAP (Cecchini et al., 2019) were measured. The relevant methodology for RT-PCR has previously been described in chapter 2 section 2.3.4.

Table 22: Genes used for real time-PCR with forward and reverse primer sequences and product length

Gene	Orientation	Primer sequence (5'-3')	Product length (BP)	Accession number
NLRP3	Forward Reverse	GAGGTCCTCTACAGCTTGTG ACATGATCATCTGTGTGGTG	127	NM_001348947.2
CASP1	Forward Reverse	TAAGCACTTGAGACAGCGGGACG GGATGTCCGTGGTCCCATTACTC	245	XP_015151421.1 AF031351.1
C3	Forward Reverse	AAGAAGCTCAGAGTGGTGCC TGCTGACTTTGGTCTCCGAC	166	NM_001396143.1
iNOS	Forward Reverse	CCACCAGGAGATGTTGAATATGTC CCAGATGTGTGTTTTCCATGCA	76	NM_204961.2
GFAP	Forward Reverse	TGTCCCTGTGCAGAGCTT CTCCTTGTGCTGCTT	157	XM_040692274.2

5.3.9 Statistical analysis

This chapter uses LBR as a reference gene where all target genes measured in HF and blood samples are to be compared in a ratio of target gene to reference gene, to normalise gene expression consistently with other studies (Dunn et al., 2013). For inflammatory cytokines in the blood analysis where we measured IL-1β, TNF-α, TGF-β and IL-6, this was repeated with anti-inflammatory cytokine IL-10 in place of LBR to understand the ratio of pro- to anti-inflammatory cytokine expression. The log of each ratio was used to calculate a Z score (Zi), to account for potential variation between RT-PCR runs.

All graphs and statistical analysis were performed using R Studio (version 1.3.959). For analysis of HF tissue and blood samples across all time points the linear mixed effects model (LMER) with repeated measures and type 3 ANOVA was performed using the AFEX package. The model included rearing type and stress group as fixed factors alongside blood group and HF as within subject factors. Bird ID was included as a random effect. There were a total of 12 birds from each group, giving a total of 48 samples. As samples were prepared and analysis was performed, these numbers do differ due to various reasons including less available RNA or issues during sample collection which are outlined in each results table. Where a significant effect was found ($p<0.05$), a pairwise comparison was performed using the estimated marginal means (EMMEANS) package. Statistical significances of $p<0.05$ are listed in text where appropriate, full statistical analysis can be found in appendix 1. All graphs were produced using GGPLOT2 in R Studio where the log10 of the ratio for each target gene has been plotted where relevant.

5.4 Results

5.4.1 Spleen mass

There was no relationship between body mass and spleen mass of all birds, nor did spleen mass change amongst control vs stress and or brooding types. Statistical data can be found in Table 27: Statistical output from the linear model with three-way ANOVA of spleen mass (g) and body mass (kg) of birds subject to UCMS or no stress in birds between rearing systems: conventional and dark brooder

5.4.2 Analysis of blood

As a first step for this study, I analysed gene expression of inflammatory cytokines in the blood across three different time points: pre-UCMS, at week 4 of UCMS and on the final week which was week 8 of UCMS. The sample size of blood obtained from time point A was significantly smaller than time points B and C due to difficulties obtaining the sample. Full statistical outputs for the linear mixed model can be found in table 5 and pair-wise analysis in table 23. This study determined there was a significant effect between rearing groups on IL-1β/IL-10 (F(1,39)=4.1568, P=0.04825). Pair-wise analysis determined that IL-1β/IL-10 expression was significantly higher in the Dark brooder group (t(38)=-2.024, P=0.0499) (figure 16).

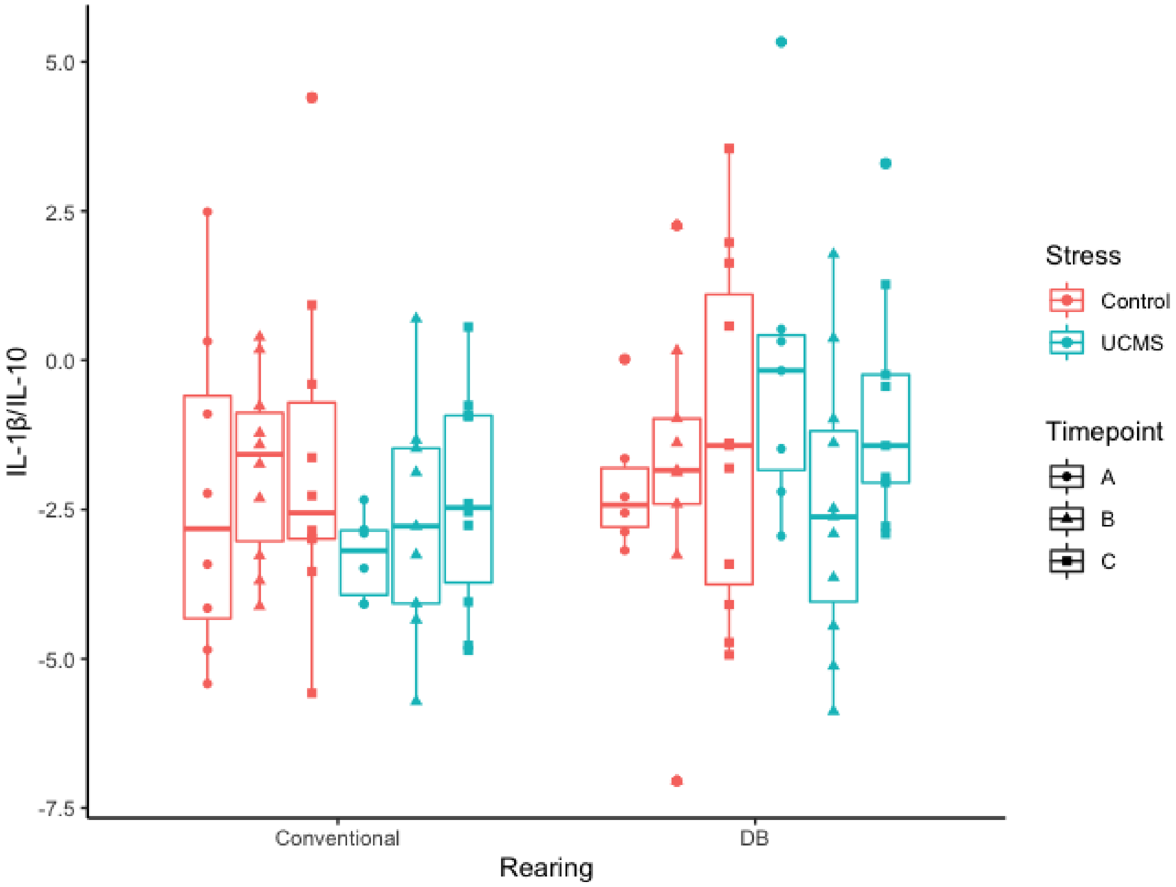

Figure 15: Gene expression of IL-1β/IL-10 in the blood across three time points (A-C) in conventional or dark brooder reared birds with UCMS or control

The ratio log of IL-1β/IL-10 gene expression in blood obtained from three different sampling time points (A, B and C) of birds subject to stress (UCMS) or no stress, from two rearing systems: conventional and dark brooder (DB). The line visualises the median of each group with the hinges representing the upper and third quartile.

Blood sample A n= 8 conventional control, 6 conventional UCMS, 6 DB control, 7 DB UCMS.
Blood sample B n= 10 conventional control, 9 conventional UCMS,9 DB control, 11 DB UCMS.
Blood sample C n= 10 conventional control, 10 conventional UCMS, 11 DB control, 9 DB UCMS.

However, there was no effect of either stress group or brooding on the expression of any other genes which can be found in table 23.

There was however a significant effect of the time the blood was sampled on the following ratios: IL-1β/LBR (F(2,52)=4.1217, P=0.02176), IL-10/LBR (F(2,98)=13.3323, P=0.0000005, TGF-β/LBR (F(2,66)=3.9585, P=0.02378), TGF-β/IL-10 (F(2,91)=17.9743, P=0.00000006) and TNF-α/IL-10 (F(2,63)=7.9972, P=0.0007947), but no effect of brooding or stress (table 23).

Table 23: Gene expression in the blood from three time points of birds with and without UCMS from two rearing types: conventional and DB

Statistical output from the linear mixed model with three-way ANOVA of gene expression in a ratio with LBR and IL-10 (Zi) of blood obtained from three-time sampling points between birds subject to stress or no stress of birds from two rearing systems: dark brooder and conventional. All significant interactions (p<0.05) are highlighted in bold.

IL-6/LBR	Time	F(2,14)=0.2337, P=0.7946
	Rearing	F(1,34)=0.0245, P=0.8766
	Stress	F(1,34)=0.0245, P=0.8766
	Time: Stress	F(2,14)=0.1820, P=0.8355
	Rearing: Time	F(2,14)=0.4171, P=0.6668
	Rearing: Stress	F(1,34)=0.2945, P=0.5909
	Time: Rearing: Stress	F(2,14)=0.1042, P=0.9017
IL-6/IL-10	**Time**	**F(2,55)=5.5928, P=0.006151**
	Rearing	F(1,55)=2.8963, P=0.094432
	Stress	F(1,48)=0.1063, P=0.74586
	Time: Stress	F(2,48)=0.2333, P=0.79285
	Rearing: Time	F(2,48)=0.0980, P=0.90682
	Rearing: Stress	F(1,48)=3.3078, P=0.07519
	Time: Rearing: Stress	F(2,48)=1.0696, P=0.35117
IL-1β/LBR	**Time**	**F(2,52)=4.1217, P=0.02176**
	Rearing	F(1,39)=0.0496, P=0.82481
	Stress	F(1,39)=0.0851, P=0.77205
	Time: Stress	F(2,52)=1.9757, P=0.14887
	Rearing: Time	F(2,52)=0.4373, P=0.64810
	Rearing: Stress	F(1,39)=0.0057, P=0.94007
	Time: Rearing: Stress	F(2,52)=0.3183, P=0.72878
IL-1β/IL-10	Time	F(2,71)=0.7578, P=0.47243
	Rearing	**F(1,39)=4.1568, P=0.04825**
	Stress	F(1,39)=0.0719, P=0.79005
	Time: Stress	F(2,71)=1.7216, P=0.18614
	Rearing: Time	F(2,71)=1.6621, P=0.19700
	Rearing: Stress	F(1,39)=2.0518, P=0.15995
	Time: Rearing: Stress	F(2,71)=0.2656, P=0.76752
IL-10/LBR	**Time**	**F(2,98)=13.3323, P=0.0000005**

	Rearing	F(1,98)=1.3096, P=0.2553
	Stress	F(1,98)=0.7444, P=0.3903
	Time: Stress	F(2,98)=0.8882, P=0.4147
	Rearing: Time	F(2,98)=1.7807, P=0.1739
	Rearing: Stress	F(1,98)=0.4525, P=0.5027
	Time: Rearing: Stress	F(2,98)=0.2227, P=0.8008
TGF-β/LBR	**Time**	**F(2,66)=3.9585, P=0.02378**
	Rearing	F(1,41)=0.0100, P=0.92085
	Stress	F(1,41)=0.0017, P=0.96777
	Time: Stress	F(2,66)=0.0418, P=0.95906
	Rearing: Time	F(2,66)=0.3697, P=0.69237
	Rearing: Stress	F(1,41)=0.6057, P=0.44078
	Time: Rearing: Stress	F(2,66)=1.8022, P=0.17295
TGF-β/IL-10	**Time**	**F(2,91)=17.9743, P=0.00000006**
	Rearing	F(1,91)=1.6525, P=0.2019
	Stress	F(1,91)=0.2203, P=0.6399
	Time: Stress	F(2,91)=0.3012, P=0.7407
	Rearing: Time	F(2,91)=1.2113, P=0.3026
	Rearing: Stress	F(1,91)=0.0000, P=0.9976
	Time: Rearing: Stress	F(2,91)=0.1255, P=0.8822
TNF-α/LBR	Time	F(2,60)=0.0999, P=0.9050
	Rearing	F(1,38)=0.3722, P=0.5454
	Stress	F(1,38)=0.8758, P=0.3552
	Time: Stress	F(2,60)=2.0552, P=0.1369
	Rearing: Time	F(2,60)=0.3840, P=0.6828
	Rearing: Stress	F(1,38)=0.4261, P=0.5177
	Time: Rearing: Stress	F(2,60)=0.6662, P=0.5174
TNF-α/IL-10	**Time**	**F(2,63)=7.9972, P=0.0007947**
	Rearing	F(1,42)=0.2004, P=0.656660
	Stress	F(1,42)=2.6230, P=0.112677
	Time: Stress	F(2,63)=0.3253, P=0.723502
	Rearing: Time	F(2,63)=1.8879, P=0.159734
	Rearing: Stress	F(1,42)=0.1419, P=0.708262

	Time: Rearing: Stress	F(2,63)=0.7403, P=0.481021

To determine the changes amongst cytokine expression between blood sampling time points, a pairwise comparison was performed for all the above significant effects and can be found in table 24. For anti-inflammatory cytokine IL-10/LBR, expression was higher in blood sampling point A, which saw a significant decrease across groups A-B and A-C, which can be observed in figure 17. IL-1β/LBR expression also significantly decreased between time points A-B, but not A-C or B-C. These birds are not to be believed to have had any recent vaccination which could have led to the increases shown in figure 17.

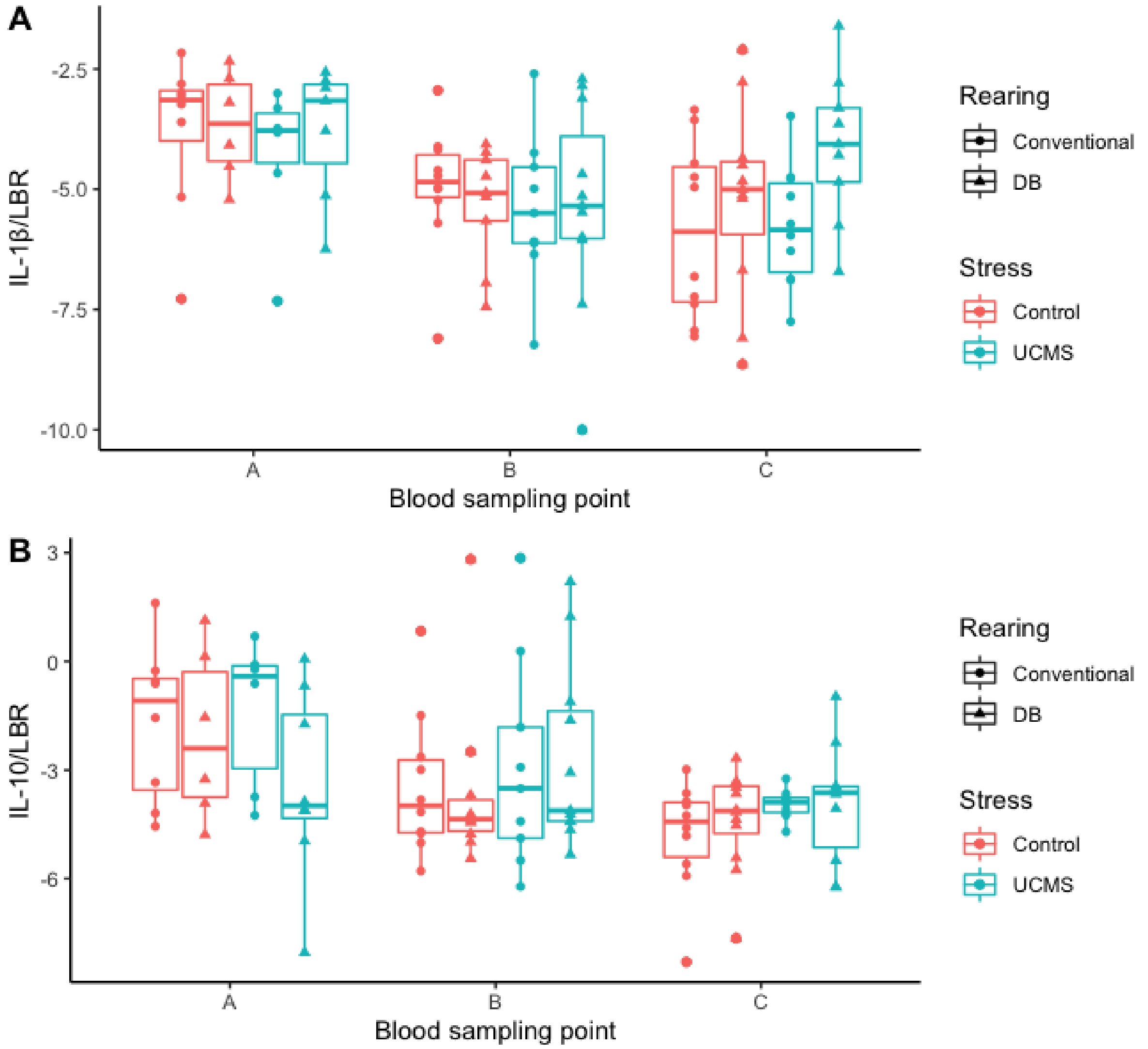

Figure 16: Gene expression of IL-1β/LBR and IL-10 LBR in the blood across three time points (A-C) in conventional or dark brooder reared birds with UCMS or control

The ratio log of IL-1β/LBR (A) and IL-10/LBR (B) gene expression in blood from three different sampling points in Dark brooder and conventionally reared birds subject to either UCMS or no stress (Control). The line visualises the median of each group with the hinges representing the upper and third quartile.

A: Blood sample A n= 8 conventional control, 6 conventional UCMS, 6 DB control, 7 DB UCMS.
Blood sample B n= 10 conventional control, 9 conventional UCMS,9 DB control, 11 DB UCMS.
Blood sample C n= 10 conventional control, 10 conventional UCMS, 11 DB control, 9 DB UCMS.
B: Blood sample A n= 8 conventional control, 6 conventional UCMS, 6 DB control, 8 DB UCMS.
Blood sample B n= 10 conventional control, 9 conventional UCMS,10 DB control, 11 DB UCMS.
Blood sample C n= 10 conventional control, 10 conventional UCMS, 12 DB control, 10 DB UCMS.

The reverse was present for inflammatory cytokines IL-6/IL-10 where expression was at its lowest in the first time point, but significantly rose between time points A-C (figure 18, table 24). Expression of TNF-α/IL-10 also increased from time points A-B and A-C, but not between time points B and C (figure 18, table 24).

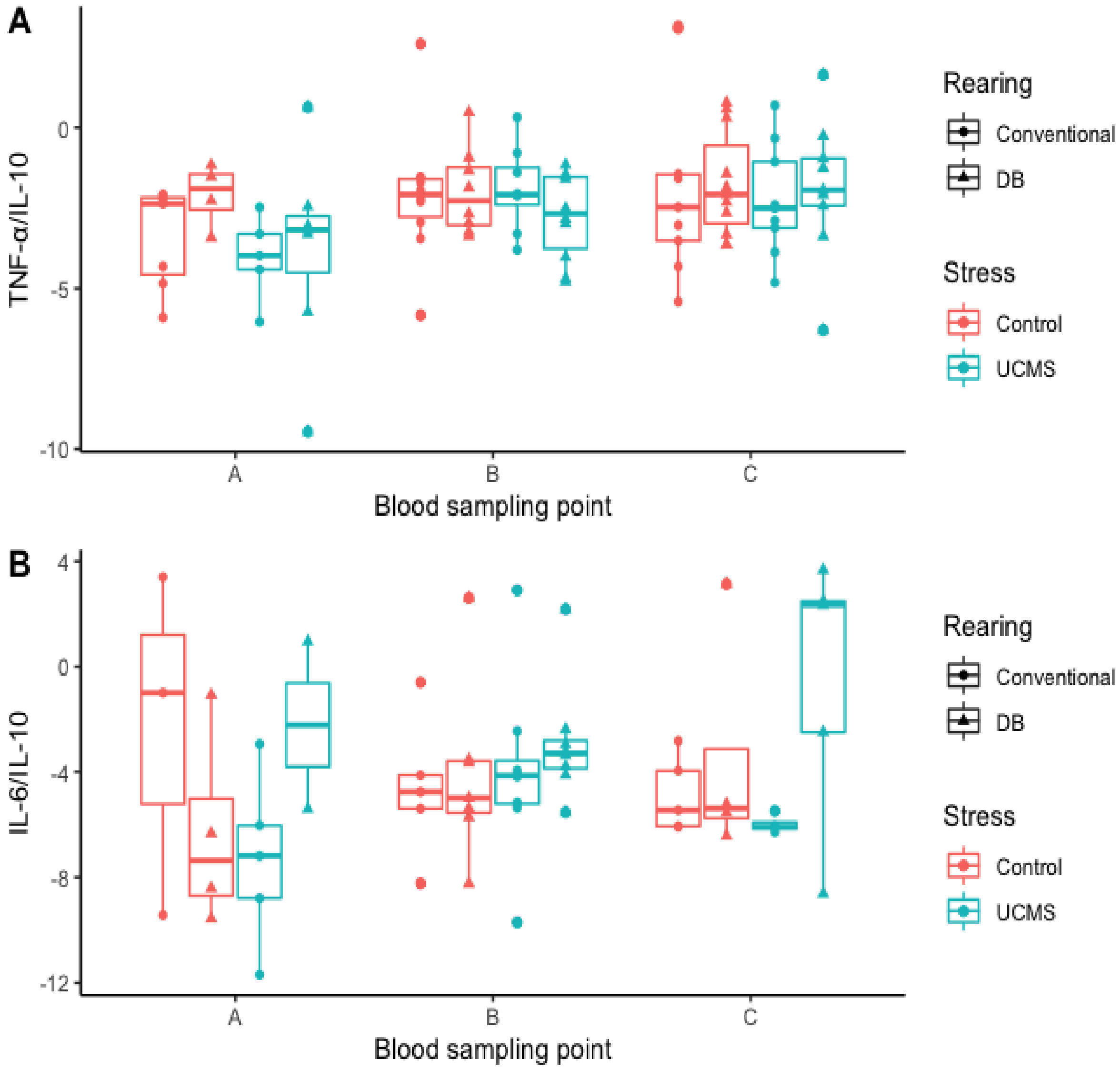

Figure 17: Gene expression of TNF-α/IL10 and IL-6/IL-10 in the blood across three time points (A-C) in conventional or dark brooder reared birds with UCMS or control

The ratio log of TNF-α/IL-10 (A) and IL-6/IL-10 (B) gene expression in blood from three different sampling points in Dark brooder and conventionally reared birds subject to either UCMS or no stress (Control). The line visualises the median of each group with the hinges representing the upper and third quartile.

A: Blood sample A n= 7 conventional control, 5 conventional UCMS, 4 DB control, 7 DB UCMS.
Blood sample B n= 10 conventional control, 8 conventional UCMS,8 DB control, 10 DB UCMS.
Blood sample C n= 9 conventional control, 9 conventional UCMS, 11 DB control, 9 DB UCMS.
B: Blood sample A n= 3 conventional control, 5 conventional UCMS, 4 DB control, 2 DB UCMS.
Blood sample B n= 5 conventional control, 8 conventional UCMS, 7 DB control, 8 DB UCMS.
Blood sample C n= 5 conventional control, 4 conventional UCMS, 4 DB control, 5 DB UCMS.

TGF-β/LBR (figure 19A) was also significantly changed between time points A-C only, where expression rose. When comparing expression of TGF-β/IL-10 (figure 19B), this time expression significantly rose between time points A-B and A-C, but not B-C (table 24).

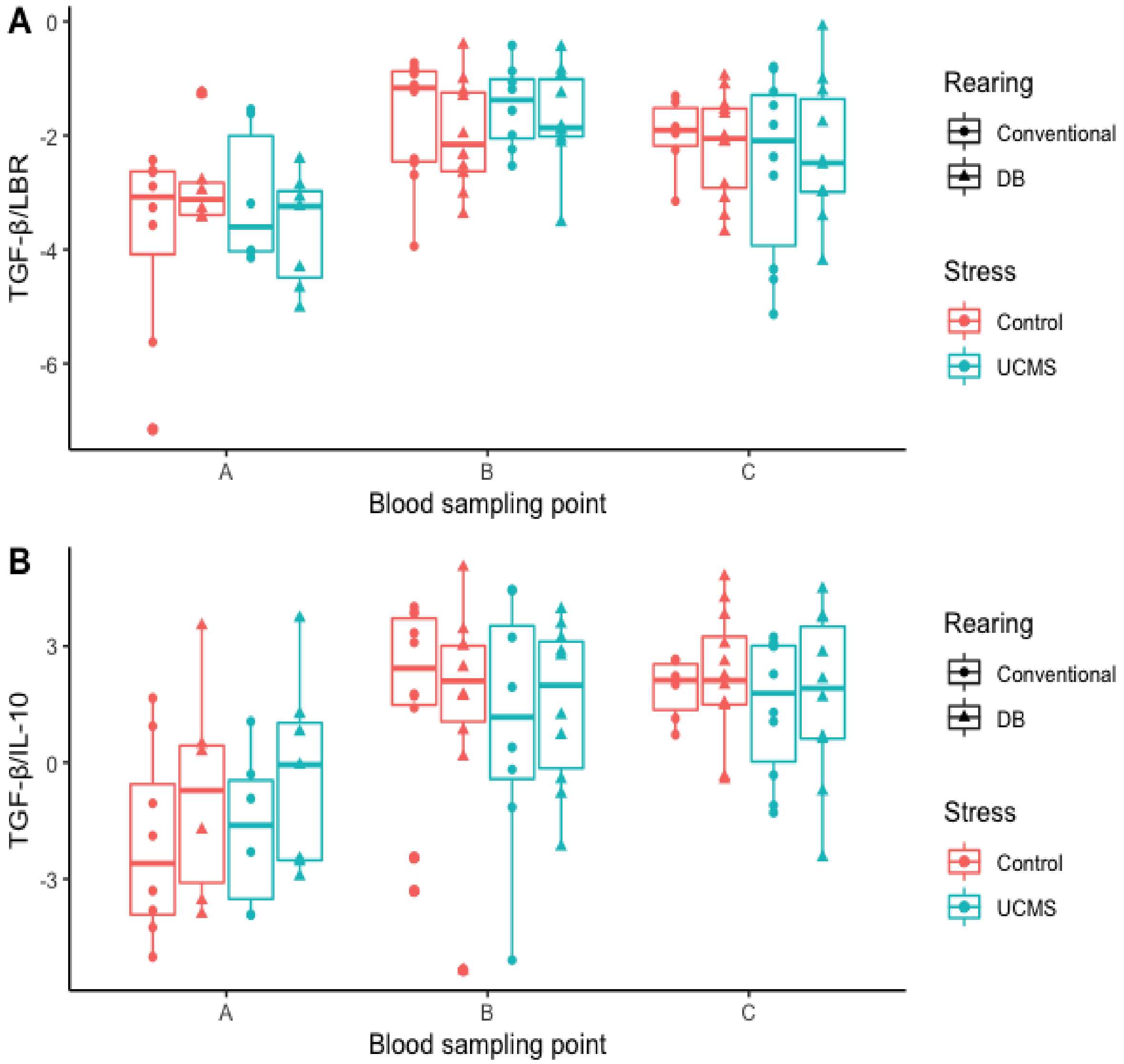

Figure 18: Gene expression of TGF-β/LBR and TGF-β/IL-10 in the blood across three time points (A-C) in conventional or dark brooder reared birds with UCMS or control

The ratio log of TGF-β/LBR (A) and TGF-β/IL-10 (B) gene expression in blood from three different sampling points in Dark brooder and conventionally reared birds subject to either UCMS or no stress (Control). The line visualises the median of each group with the hinges representing the upper and third quartile.

Blood sample A n= 8 conventional control, 6 conventional UCMS, 6 DB control, 7 DB UCMS.
Blood sample B n= 10 conventional control, 8 conventional UCMS, 10 DB control, 10 DB UCMS.
Blood sample C n= 6 conventional control, 10 conventional UCMS, 12 DB control, 10 DB UCMS.

Table 24: Estimated marginal means of gene expression in the blood from three time points of birds with and without UCMS from two rearing types: conventional and DB

Pair-wise analysis output from the estimated marginal means of all significant interactions from the linear mixed model with three-way ANOVA of gene expression (Zi) in the blood from three different sampling points: A-B, A-C and B-C.

IL-6/IL-10	A-B	T(49)= -2.339, P=0.0597	-
	A-C	**T(53)= -3.204, P=0.0064**	**Increase**
	B-C	T(45)= -1.261, P=0.4244	**-**
IL-1β/LBR	**A-B**	**T(57)= 2.852, P=0.0164**	**Decrease**
	A-C	T(54)= 1.552, P=0.2752	**-**
	B-C	T(57)= -1.529, P=0.2851	**-**
IL-10/LBR	**A-B**	**T(76)= 0.439, P=0.0027**	**Decrease**
	A-C	**T(74)= 5.105, P=0.0001**	**Decrease**
	B-C	T(72)= 1.815, P=0.1720	**-**
TGF-β/LBR	**A-B**	**T(65)= -2.730, P=0.0219**	**Increase**
	A-C	T(67)= -2.072, P=0.1033	**-**
	B-C	T(63)= 0.672, P=0.7807	**-**
TGF-β/IL-10	**A-B**	**T(69)= -5.245, P=0.0001**	**Increase**
	A-C	**T(73)= -5.314, P=0.0001**	**Increase**
	B-C	T(67)= -0.156, P=0.9867	**-**
TNF-α/IL-10	**A-B**	**T(63)= -2.430, P=0.0466**	**Increase**
	A-C	**T(61)= -3.958, P=0.0006**	**Increase**
	B-C	T(60)= -1.731, P=0.2021	-

5.4.3 Brain analysis

Next, I measured gene expression across the rostral and caudal HF (table 25). First to determine differences in the expression of microglia cells I measured CSFR-1/LBR which was not significantly different across any treatment groups. Then I measured astrocyte marker GFAP/LBR which there was a significant effect between the rostral and caudal HF (F(1,43)=23.5267, P=0.0000059) across all groups where pair-wise analysis show GFAP is expressed at greater quantities in the caudal HF (t(43)=4.89, P=0.0001) (table 26, figure 20). There was however a significant two-way interaction between HF and stress groups F(1,43)=4.2755, P=0.04460). Pair-wise comparison confirms GFAP is higher in the caudal than in the rostral HF of birds subject to UCMS only (t(43)=4.771, P=0.0001) (table 25, table 26).

Table 25: Gene expression in the rostral and caudal HF of birds with and without UCMS from two rearing types: conventional and DB

Statistical output from the linear mixed model with three-way ANOVA of gene expression in a ratio with LBR (Zi) in the Rostral and Caudal HF of all treatment groups. All significant effects (p<0.05) are highlighted in bold.

iNOS/LBR	**Rostrocaudal**	**F(1,45)=7.4324, P=0.009096**
	Rearing	F(1,44)=2.8685, P=0.097398
	Stress	F(1,44)=0.3163, P=0.576707
	Rostrocaudal: Stress	F(1,45)=2.3384, P=0.133215
	Rostrocaudal: Rearing	F(1,43)=1.5017, P=0.227082
	Rearing: Stress	F(1,43)=0.2745, P=0.603013
	Rostrocaudal: Rearing: Stress	F(1,43)=0.0520, P=0.820702
GFAP/LBR	**Rostrocaudal**	**F(1,43)=23.5267, P=0.0000059**
	Rearing	F(1,44)=0.0289, P=0.86584
	Stress	F(1,44)=0.0003, P=0.98605
	Rostrocaudal: Stress	**F(1,43)=4.2755, P=0.04460**
	Rostrocaudal: Rearing	F(1,43)=3.8318, P=0.05667
	Rearing: Stress	F(1,43)=0.2489, P=0.62039
	Rostrocaudal: Rearing: Stress	F(1,42)=0.3487, P=0.55798
CSFR-1/LBR	Rostrocaudal	F(1,43)=2.4918, P=0.1218
	Rearing	F(2,57)=0.3153, P=0.7308
	Stress	F(1,43)=0.5481, P=0.4631
	Rostrocaudal: Stress	F(1,43)=0.2578, P=0.6143
	Rostrocaudal: Rearing	F(1,43)=0.0538, P=0.8177
	Rearing: Stress	F(1,43)=0.8827, P=0.3527
	Rostrocaudal: Rearing: Stress	F(1,43)=0.0515, P=0.8215
C3/LBR	Rostrocaudal	F(1,43)=0.0748, P=0.785775
	Rearing	F(1,41)=2.0531, P=0.159424
	Stress	**F(1,41)=11.4401, P=0.00158**
	Rostrocaudal: Stress	F(1,40)=2.7526, P=0.104806
	Rostrocaudal: Rearing	F(1,40)=0.4261, P=0.517590
	Rearing: Stress	**F(1,41)=4.2996, P=0.044398**
	Rostrocaudal: Rearing: Stress	F(1,40)=0.1855, P=0.668954
CASP-1/LBR	Rostrocaudal	F(2,80)=0.1572, P=0.8548

	Rearing	F(1,80)=1.0736, P=0.3033
	Stress	F(1,80)=0.1689, P=0.6822
	Rostrocaudal: Stress	F(1,80)=0.0688, P=0.7937
	Rostrocaudal: Rearing	F(1,80)=0.5961, P=0.4423
	Rearing: Stress	F(1,80)=2.4095, P=0.1246
	Rostrocaudal: Rearing: Stress	F(1,80)=2.7077, P=0.1038
NLRP-3/LBR	Rostrocaudal	F(2,82)=1.0841, P=0.3430
	Rearing	F(1,82)=0.3735, P=0.5428
	Stress	F(1,82)=1.0540, P=0.3076
	Rostrocaudal: Stress	F(1,82)=0.0849, P=0.7715
	Rostrocaudal: Rearing	F(1,82)=2.1863, P=0.1431
	Rearing: Stress	F(1,82)=0.6050, P=0.4389
	Rostrocaudal: Rearing: Stress	F(1,82)=0.1308, P=0.7185

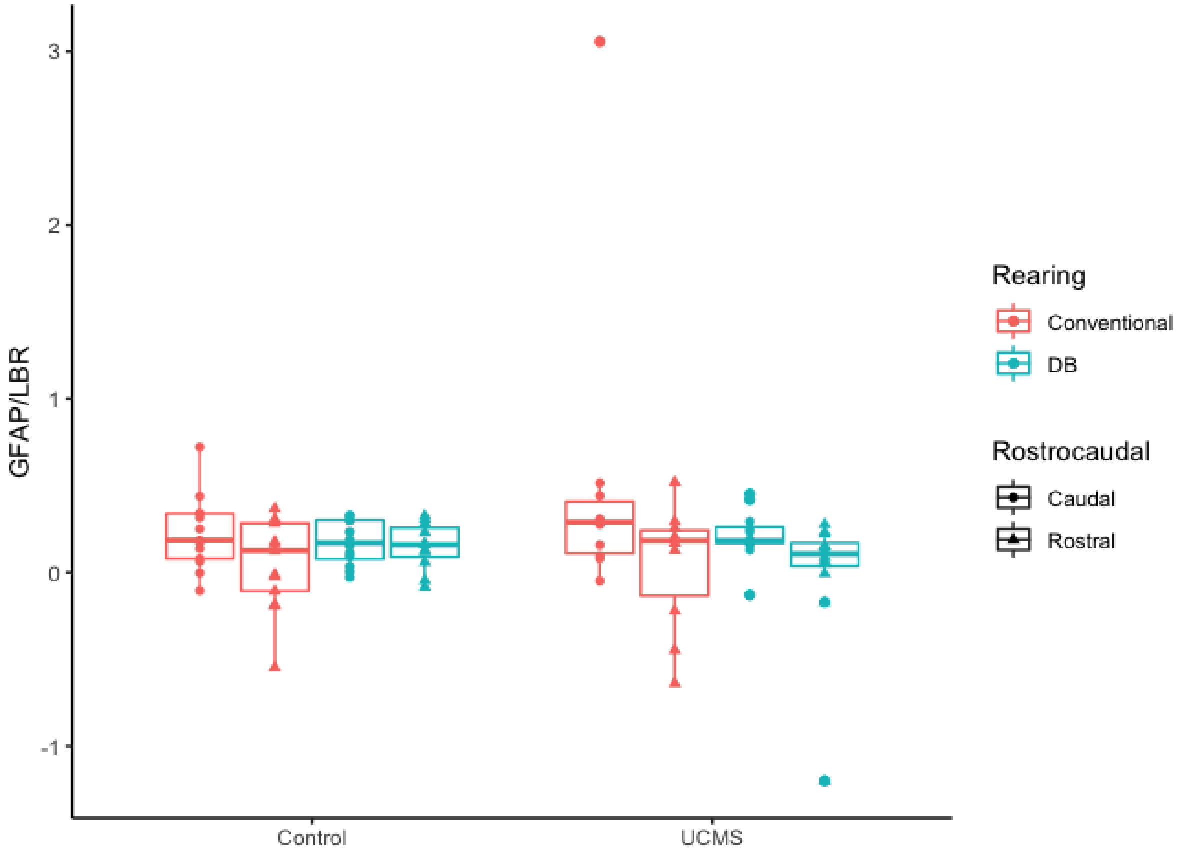

Figure 19: Gene expression of GFAP/LBR in the rostral and caudal HF of birds from conventional or dark brooder rearing with UCMS or control

The ratio log of GFAP/LBR gene expression in the rostral and caudal HF of Dark brooder and conventionally reared birds subject to UCMS or no stress (UCMS). The line visualises the median of each group with the hinges representing the upper and third quartile. Sample n= conventional control: 13 rostral 13 caudal, conventional UCMS: 10 rostral 10 caudal, DB control: 11 rostral 12 caudal, DB UCMS: 12 rostral 12 caudal.

To determine the activation status of astrocytes, a group of markers were selected. Firstly, I measured iNOS expression where again there was a significant effect between rostral and caudal HF in all birds (F(1,45)=7.4324, P=0.009096) (table 25). Pair-wise comparison revealed iNOS is expressed at greater levels again in the caudal HF (t(43)=2.571, P=0.0087) (table 26). There were no statistical differences across all groups in CASP-1 or NLRP-3 indicating no effect of treatment or anatomical differences of these genes (table 25). However, UCMS birds had higher C3 expression than controls (F(1,41)=11.4401, P=0.00158). Stress also interacted significantly with rearing group (F(1,41)=4.2996, P=0.044398) (table 25). The increase in C3 expression in UCMS birds compared to control only occurred in the conventional group (t(41)=-3.17, P=0.0033), and not in the dark brooder group (table 26, figure 21). There were no differences in C3 expression between the rostral vs caudal HF.

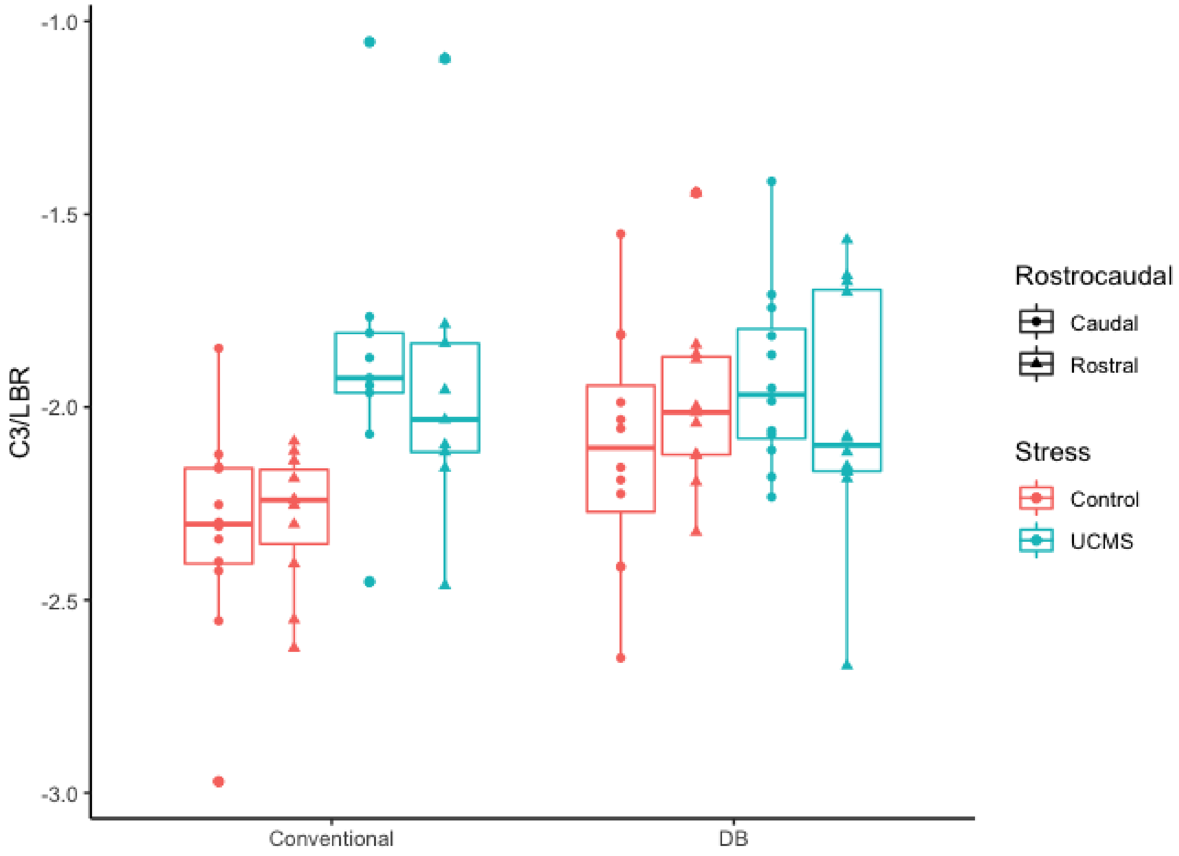

Figure 20: Gene expression of C3/LBR in the rostral and caudal HF of birds from conventional and dark brooder rearing with UCMS or control

The log ratio of C3/LBR gene expression in the rostral and caudal HF of Dark brooder and conventionally reared birds, subject to UCMS or no stress (Control). The line visualises the median of each group with the hinges representing the upper and third quartile. Sample n= conventional control: 11 rostral 12 caudal, conventional UCMS: 9 rostral 9 caudal, DB control: 11 rostral 12 caudal, DB UCMS: 12 rostral 12 caudal.

Table 26: Estimated marginal means of gene expression in the rostral and caudal HF of birds with and without UCMS.

Pair-wise analysis output from the estimated marginal means of all significant interactions from the linear mixed model with three-way ANOVA of gene expression (Zi) in the Rostral and Caudal HF.

GFAP/LBR	Caudal Control – Rostral Control	t(44)= 2.009, P=0.2002	-
	Caudal Control – Caudal UCMS	t(74)= -1.132, 0.6710	-
	Caudal Control – Rostral UCMS	t(74)= 2.599, P=0.0538	-
	Rostral Control – Caudal UCMS	t(75)= -2.606, P=0.0528	-
	Rostral Control – Rostral UCMS	t(75)= 1.092, P=0.6956	-
	Caudal UCMS – Rostral UCMS	**t(43)= 4.771, P=0.0001**	**Caudal increase**
	Caudal - Rostral	**t(43)= 4.849, P=0.0001**	**Caudal increase**
C3	Conventional Control – DB Control	t(42)= -2.562, P=0.651	-
	Conventional Control – Conventional UCMS	**t(41)= -3.718, P=0.0033**	**Conventional UCMS increase**
	Conventional Control – DB UCMS	**t(41)= -3.541, P=0.0054**	**DB UCMS increase**
	DB Control – Conventional UCMS	t(41)= -1.329, P=0.5503	-
	DB Control – DB UCMS	t(41)= -0.963, P=0.7711	-
	Conventional UCMS – DB UCMS	t(40)= 0.439, P=0.9713	-
	Control - UCMS	**t(41)= -3.381, P=0.0016**	**UCMS increase**

5.5 Discussion

5.5.1 Expression of inflammatory cytokines in the blood during UCMS

This study used UCMS in hens which has been associated with reduced hippocampal plasticity and hypothesised that inflammatory cytokines would be increased during the initial stages of chronic stress, which subsequently leads to neuroinflammation including activation of astrocytes. This chapter predicted that if inflammation is involved with chronic stress, there would be a gradual increase in inflammatory cytokines in the blood between sampling time points of the UCMS group of birds housed in conventional housing without DB rearing. In addition, there would be an increase of astrocyte marker GFAP and inflammation-related genes measured in this study. This study hypothesised that hens reared with DB would be less susceptible to the effects of UCMS with no increase in the genes measured between UCMS and control.

This chapter measured gene expression of inflammatory cytokines IL-1β, IL-6, TNF-α and TGF-β alongside anti-inflammatory IL-10 in the blood from birds subject to UCMS or no stress from three time points: pre-UCMS, 4 weeks in and at 8 weeks as the protocol came to an end. Results show a significant effect of rearing condition on IL-1β/IL-10 where expression was increased in the dark brooder birds at all time points with no effect of stress (figure 16, table 23). This result might suggest hens reared as dark brooders express greater levels of IL-1β/IL-10 (pro-inflammatory to anti-inflammatory) in the blood which does not change between UCMS or control birds. The reason for this is unclear, however, dark brooding is thought to provide additional nurturing early on which could have a positive effect on the immune system which is still developing during the first 3 weeks of life. Although when each cytokine was compared independently with the control gene LBR, there were no significant changes between rearing groups and the ratio of IL-1β/IL-10 was only just significant and could be spurious (table 23).

Table 6 shows that when all genes excluding IL-1β/IL-10 and IL-10/LBR were measured in the blood across the three time points, there was an increase in expression and decreased IL-10/LBR throughout the study which affected all birds. However, there were sampling limitations for blood time point A, where difficulty obtaining blood upon the first attempt resulted in a smaller sample size. These birds had no known infections or recent vaccinations, suggesting systemic inflammatory cytokines increase as the bird ages, which is known as inflamm-aging within mammalian research (Xia et al., 2016). As birds age and begin to lay eggs, the immune system is affected. One study in hens found as hens age from weeks 15/16 to 23/24 there is an increase in innate immune cells, but decreased peripheral and splenic lymphocytes (Schmucker et al., 2021). Inflammatory cytokines including IL-1, IL-6 and TNF-α which increased with age in this study, are secreted by innate immune cells (Kogut, 2000). The increase in the cytokines measured in this study which occurred as the hens aged is consistent with studies in animal experiments demonstrating elevated plasma expression of IL-1β and TNF-α in elderly horses (Adams et al., 2008) and IL-1, IL-6 and TNF-α in elderly patients (Bruunsgaard et al., 2003). Therefore it is likely the increase in pro-inflammatory cytokines in the blood observed in all hens aged 16 weeks to 25 is due to the age of the bird or even puberty rather than an implication of this study or a transient increase of cytokine expression similar to findings in a rodent model of repeated water avoidance stress who found a time dependent increase in IL-1β (Glovatchcka et al., 2008). This chapter investigated if the expression of these cytokines varied throughout the stress response, as in previous chapters there was no change in expression between stress and no stress at the end of the study. As stress did not influence gene expression in the blood during all three time points, the time points cytokine expression was measured during chronic stress might not have been the right approach. Due to limit sample availability, this study was not able to measure cytokine expression in the spleen or hippocampus and relied on blood sampling. As found in chapter 3, IL-1β was increased in the HF of birds with newer fractures and a systemic increase was not investigated. Therefore, it is unclear whether inflammatory cytokines including IL-1β increase in the early stages of UCMS in the hippocampus or where they are produced, which subsequently may lead to reduced neuronal plasticity.

5.5.2 Microglia activation marker CSFR-1 in the HF is not increased during UCMS

Secondly this chapter measured mRNA in the rostral and caudal HF of CSFR-1 gene expression and consistently with chapters 1 and 2 which measured CSFR-1, there was again no significant differences in expression between UCMS birds and control, alongside no differences between conventional and DB birds. To further explore the potential involvement of neuroinflammation in stressed hens this chapter measured gene expression of further markers associated with neuroinflammation including iNOS which is produced by ameboid microglia to secrete nitric oxide when stimulated with LPS in the quail retina (Sierra et al., 2014) which was also not impacted by UCMS.

5.5.3 Neurotoxic A1 Astrocytes in the HF during UCMS

Next this chapter measured a profile of genes which have been associated with neurotoxic astrocytes known as A1, which began with the general astrocyte marker GFAP to measure changes in the total of astrocyte cells. GFAP was increased in the caudal HF and lower in the rostral of all birds who received UCMS, with no anatomical difference in the control birds (table 25, table 26). This result indicates that when the hens are under UCMS, there is increased astrocyte presence in the caudal HF which less in control birds (Figure 20). In line with a study which investigated A1 astrocytes through commonly used markers $GFAP^{+}C3^{+}$ (S. Li et al., 2022), this study then measured gene expression of C3 in the rostral and caudal HF. Results show C3 expression is increased in all birds who received UCMS treatment compared to control birds overall, however there was a further difference in expression amongst the conventional vs DB hens (figure 21, table 25). Although DB birds who received UCMS treatment had significantly higher C3 expression than conventionally reared birds with no UCMS, there was no difference in C3 expression between conventional birds with UCMS and control DB birds. This result suggests in the absence of UCMS, birds reared as DB have comparable levels of C3 than the birds who were conventionally reared with UCMS. This result indicates two possibilities: 1) Birds with UCMS treatment have increased GFAP expression in the caudal HF and increased C3 across the whole HF and 2) C3 expression is increased on this occasion in birds who were reared as DB with and without UCMS compared to conventionally reared birds with no UCMS treatment. These findings are consistent with (S. Li et al., 2022) who also found increased $GFAP^{+}C3^{+}$ astrocytes at two-weeks into chronic mild stress throughout the hippocampus, but reported lower overall $GFAP^{+}$ cells in this group which was not the case in the present chapter. This does suggest an increase of $GFAP^{+}C3^{+}$ astrocytes under chronic stress, however this chapter measured mRNA gene expression and (S. Li et al., 2022) used immunohistochemistry techniques. The drawback of measuring gene expression is that an increase in gene expression does not translate to the total C3 being secreted, or enable any morphological analysis. This chapter only found increased GFAP gene expression in the caudal HF and increased C3 across the whole HF under UCMS which can only indicate the presence of A1 astrocytes rather than confirm the increased presence of these C3 producing astrocytes as immunohistochemistry could, nor can it confirm it is the astrocytes directly producing C3. C3 is just one of many complement proteins, however, C3 is the convergence point during activation for all 3 complement pathways and has roles including host defence from pathogens and degeneration (Bourel et al., 2021; Ehrnthaller et al., 2013). In mammalian research, C3 has been associated astrocytes and microglia, where their typical function involves neuronal migration (Bourel et al., 2021; Zhou et al., 2023). As this chapter found an increase in both astrocyte marker GFAP and C3, it is likely that C3 is coming from astrocytes within the HF. However, their potential role in reduced hippocampal plasticity during chronic stress is still unclear.

Previous work in hens treated with UCMS found there was a suppression of incorporation of DCX$^+$ neurons in the caudal HF, which was thought to be homologous to the ventral HF in mammals (Gualtieri et al., 2019). With that said, a study in wild-type mice found GFAP$^+$ astrocytes share different characteristics between the dorsal and ventral HF (Viana et al., 2023). This study suggest astrocytes in the ventral HF were structurally more ramified with additional and longer processes, which could explain why caudal GFAP gene expression is increased in this chapter, but why this was strengthened during UCMS is unclear. One study in rats discovered activity stress (AS) for 6 days lead to a 30% increase in GFAP-immunoreactive cells in the hippocampal CA3 region in comparison to the control group (Lambert et al., 2000), although this was considered to be an increase of GFAP levels rather than an increase in the number of glial cells. The study which found the time dependent increase of IL-1β in the blood of rats, who faced WA also found that GFAP expression initially increases at 24 hours post 5 consecutive days of WA, to then decrease at 24 hours following 10 consecutive days (Glovatchcka et al., 2008).

This chapter reported increased C3 gene expression across the rostral and caudal HF in all birds facing UCMS compared to control. Upregulated C3 expression in the denate gyrus of the mouse hippocampus has been observed in experimental multiple sclerosis alongside reduction of total dendritic length, although this increase was microglia producing C3 (Bourel et al., 2021). When this same study assessed microglial engulfment of the post-synaptic protein Homer in CD86-positive lysomes they found significantly higher lysosomal homer puncta in microglia cells within the experimental encephalomyelitis (EAE) group, suggesting hippocampal dendritic and synaptic loss in this study is mediated through microglial phagocytosis through a complement-dependent mechanism (Bourel et al., 2021). Increased C3 has also been associated with major depressive disorder where a mouse model of depression suggest increased C3 in the prefrontal cortex is a consequence of chronic stress (Crider et al., 2017). Whether this is the case in the present chapter remains unclear as there were no differences in CSFR-1 expression amongst UCMS and control birds. However, increased C3 expression and reduced neuron density in the HF of hens from various stress conditions (Gualtieri et al., 2019; Armstrong et al., 2022, 2020) indicate it could be a likely scenario in hens where the effect of stress on neurogenesis is through a complement-dependent mechanism.

5.5.4 Gene expression between DB reared birds and conventionally reared birds during UCMS

One final point of discussion in this chapter was the increased ratio of IL-1β/IL-10 in the blood and comparable levels of C3 in the hippocampus between conventionally raised birds facing UCMS to DB reared birds with no stress. C3 expression also did not differ between DB birds with and without UCMS as it did compared to conventionally reared birds. There is currently no research to suggest hens reared as DB have increased expression of these markers and there is no measurement of DCX^+ cell number for this study, so the meaning of this result and if this has any involvement with neurotoxicity remains unclear. As mentioned, this work was run in parallel with another study (Craven, 2024) with these same birds and gene expression of DCX and BDNF was measured. That study found that within the caudal HF of DB birds only DCX gene expression was higher in the UCMS group than control birds, with no change in the rostral HF. Furthermore, BDNF expression was this time higher in the rostral HF of all birds and there was no difference between UCMS and control DB reared birds. Although there was a change within the conventionally reared birds where BDNF is lower in UCMS birds. These findings by Craven, 2024 alongside the findings of this chapter suggest birds reared as DB respond differently to the UCMS protocol compared to conventionally reared birds. Although measuring gene expression of DCX isn't as informative as measuring cell density of DCX^+ cells due to gene expression not necessarily meaning the protein is being expressed, previous findings found DCX$^+$ cell density is decreased following UCMS in conventional birds (Gualtieri et al., 2019).

In the birds used for this chapter C3 expression was increased and BDNF expression (Craven, 2024) was decreased in conventional birds with UCMS where both markers were higher in DB vs conventional birds with no difference between UCMS and control with higher DCX for UCMS. Together these results indicate that whilst stress impacts the expression of these markers in the absence of DB rearing, when birds are reared with DB they do not experience these changes during stress. Early life stress is said to have several effects on the immune system as it is not fully developed at birth, making it possible to have effects on long-term immunity (Danese & J Lewis, 2017). Evidence does suggest hens reared as DB's could be less fearful than hens reared without any maternal support like the conventional system. It could be possible these hens are less likely to feel fear-related stress than hens without DB and could be less likely to lead to stress-induced inflammation (Barnett et al., 1992).

It is more likely that the conventional hens who are without that maternal support such as warmth and shelter are to face early life stress which could be why the results in this chapter suggest C3 and BDNF gene expression was altered in the conventional birds facing UCMS, but not the DB birds. In mammals it is generally accepted that early-life adversity leads to vulnerability and mental illness which in turn can initiate neuroinflammation (Ganguly & Brenhouse, 2015). One study in a rat model of maternal separation of male and females found IL-1β was increased in the prefrontal cortex of the brain (Abelaira et al., 2022) and another found an increase in IL-6 (Abelaira et al., 2021). Another study in rats using maternal separation combined with restraint stress found BDNF gene expression to be decreased in the hippocampus compared to controls (Seo et al., 2016). This chapter did not measure inflammatory cytokine expression in the HF as in previous chapters there was no differences in expression between the groups where DCX$^+$ density was decreased and the controls (Armstrong et al., 2022, 2020; Gualtieri et al., 2019) and there was only a slight difference in IL-1β/IL-10 expression between rearing systems in the blood. However, as newly acquired KBF lead to increased IL-1β in the HF of chapter 3, perhaps during the early stages of UCMS there might be a similar pattern showing increased cytokine expression local to the HF during early UCMS. Although if IL-1β and IL-6 were measured in the HF of DB birds, it would be interesting to see if this too is altered similar to the C3 increase and BDNF decrease in comparison to the conventional birds.

It is likely that the differences in C3 and BDNF expression between conventional UCMS and control are a result of greater susceptibility to stress caused by earlier stresses during their rearing which was not present for the DB birds. As DCX gene expression increases when DB birds face UCMS, these birds do not appear to experience the same decrease conventionally reared birds do in various studies (Armstrong et al., 2022, 2020; Gualtieri et al., 2019) which was unchanged in the conventional birds of the present chapter.

5.6 Concluding remarks

To conclude, this chapter used two different rearing systems: conventional and dark brooders to compare gene expression of neuroinflammatory markers between hens facing UCMS or control. There was only a natural increase in the inflammatory cytokines in blood measured over the 3 time points with no effect of rearing or stress. There was however an increase of GFAP gene expression in the caudal HF which was only present in all birds with UCMS treatment. This suggests altered astrocyte presence and C3 increased expression in UCMS birds compared to control, although this was more prevalent in the conventional birds. Although there was an overall increase of C3 in all stress birds, the effect was greater in the conventional birds and DB control birds had similar levels of C3 to UCMS conventional birds. BDNF was also lower in the conventional UCMS birds than control, but not in the DB birds alongside a DB increase of DCX during UCMS treatment. These results suggest that conventionally reared birds have decreased BDNF and increased C3 and caudal GFAP expression during UCMS which the DB do not, suggesting these two rearing systems lead to different stress responses.

5.7 Appendix 3

Table 27: Statistical output from the linear model with three-way ANOVA of spleen mass (g) and body mass (kg) of birds subject to UCMS or no stress in birds between rearing systems: conventional and dark brooder

Spleen mass	Body Mass	F(1,43)=1.3981, P=0.2435
	Rearing	F(1,43)=0.0000, P=0.9967
	Stress	F(1,43)=0.2088, P=0.6500
	Rearing: Stress	F(1,43)=0.2627, P=0.6109

CHAPTER 6: GENERAL DISCUSSION

6.1 Summary of research

This thesis explored the potential role of the immune system in the hippocampal formation (HF) of laying hens where reduced adult hippocampal plasticity was observed as a consequence of chronic stress. As a first step, hippocampal and spleen tissue were obtained from a study of hens where a reduction in doublecortin (DCX) cell density was observed in birds in poor physical condition from two housing systems and another involving hens with fractures of the keel bone (Armstrong et al., 2022, 2020). Following on from that, this thesis obtained further hippocampal tissue and blood samples from three time points from birds subjected to unpredictable chronic mild stress (UCMS).

The first aims of this research were to measure inflammation-related genes in spleen tissue to explore a systemic immune response to chronic stress and a local immune response in the HF. This analysis was carried out in chapters 2+3 by measuring gene expression through real-time quantitative PCR (RT-qPCR) of inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β, alongside anti-inflammatory cytokine IL-10. All genes were repeated in the rostral and caudal HF in addition to colony stimulating factor receptor-1 (CSFR-1) and brain derived neurotrophic factor (BDNF). Next in chapter 4, this research measured immunoreactive cell density of ionized calcium binding adaptor molecule-1 (IBA-1) which is a commonly used marker for microglia cells. This chapter set to measure cellular density of three morphologies of microglia cells in the HF, which each respond to a different microglia phenotype. Finally in chapter 5, this thesis repeated the inflammatory genes measured in the spleen in chapters 2+3, this time in blood tissue from three time points during unpredictable chronic mild stress (UCMS) to determine if inflammatory cytokines increase transiently during chronic stress. This was in conjunction with measuring gene expression of several markers secreted by inflammatory microglia and astrocytes in the HF.

The following sections will discuss the findings of this thesis and its implications and if there could be any establishment of a link between chronic stress induced reduced hippocampal plasticity and inflammation.

6.2 Gene expression of inflammatory cytokines are not increased in the spleen and blood during chronic stress

Previous studies in our lab established a link between chronic stress and the reduction in DCX$^+$ neurons in the HF of laying hens. This included birds with poor physical body condition (Armstrong et al., 2022) and birds with severe keel bone fractures (KBF) (Armstrong et al., 2020). The author of those two studies suggested the reduction in DCX$^+$ representing reduced hippocampal plasticity is a consequence of chronic stress. It is known from mammalian research that inflammatory cytokines are increased during periods of chronic stress. This can be triggered by circulating glucocorticoid (GC) hormones which are increased as part of activation of the hypothalamic pituitary adrenal (HPA) axis during chronic stress (Liu et al., 2017). One study in a depressive-like state mouse model which induced chronic stress by UCMS, found an increase of inflammatory cytokines IL-1β, IL-18 and TNF-α in peripheral blood after 4 weeks (Peng et al., 2012). This was accompanied by increased depressive-like behaviour, similarly to the hens with KBF who also had an onset of depressive-like state as indicated by the DCX$^+$ reduction at 4 weeks after a fracture was identified (Armstrong et al., 2020). Although Peng et al 2012 did not investigate the reduction of DCX$^+$ density in the hippocampus because of UCMS, it could be possible that the inflammatory cytokines measured are involved. Therefore, chapter 2 of this thesis set to explore the possibility of a similar increase in inflammatory cytokines in hens with reduced hippocampal plasticity.

Firstly, due to sampling limitations the spleens were only obtained from the birds from the multi-tier and colony housing systems as outlined in chapter 2, where hippocampal plasticity was reduced in birds with poor physical condition such as reduced feather coverage compared to good condition independently of housing system. Spleens were not sampled from the study looking at KBFs (chapter 3). This means there were limitations in regarding the birds with KBFs and reduced hippocampal plasticity and depressive-like behaviour, as it would have been interesting to determine if the cytokines measured in the spleen in this thesis including IL-1β were upregulated similarly to the rodents with UCMS and depressive-like behaviour (Peng et al., 2012). Nonetheless, where spleens were available in chapter 2, it was determined there was no increase in the gene expression of any inflammation-related genes including IL-1β, IL-6 and IL-10. This result indicates that where poor physical condition led to reduced hippocampal plasticity measured by reduced DCX$^+$ density, there was no increase of inflammatory cytokines in the spleen at the same time point.

The rodent UCMS study (Peng et al., 2012) did not investigate hippocampal plasticity, but UCMS has been observed to reduce DCX$^+$ density in the caudal HF of hens in a homologous way to mammals (Gualtieri et al., 2019). Therefore, in chapter 5 of this thesis the peripheral immune response was revisited by measuring inflammatory cytokines IL-1β, IL-6, TNF-α and TGF-β alongside anti-inflammatory IL-10 over 3 time points of UCMS in the blood. In chapter 5 I measured the expression of these genes in the blood on week 1 of UCMS as the experiment was starting, mid-way on week 4 and as UCMS concluded on week 8. The predictions for this chapter were that inflammation could be transient or acute and the expression of inflammatory cytokines could fluctuate throughout chronic stress. One study in male human participants which performed the Trier Social Stress Test before collecting blood at baseline, 30, 60 and 90 minutes after the test concluded. There was an immediate increase of IL-1β after 30 minutes which returned to baseline levels by 60 and 90 minutes post-test (Yamakawa et al., 2009). This is an interesting finding, as the blood obtained from birds in chapter 5 was obtained after 30 minutes in a bag, which is an acute stressor. Therefore IL-1β gene expression in chapter 5 could be already be increased for this same reason, as blood was not obtained before the acute stressor in a bag. IL-1β in plasma has again been shown to increase in a time dependent manner in a rodent model of repeated water avoidance stress (Glovatchcka et al., 2008). However, during each time point measured, there was no increase of any inflammatory genes measured, including IL-1β in the blood of the hens with UCMS compared to control. These findings in chapter 5 suggest inflammatory cytokines do not fluctuate during UCMS in hens, similarly to in chapter 2 where there was no increased expression in the spleen. Therefore, this thesis did not establish any link between inflammatory and anti-inflammatory cytokine gene expression in the periphery and hens facing chronic stress and reduced hippocampal plasticity.

6.3 Gene expression of inflammatory cytokines in the Hippocampal Formation during chronic stress

Secondly this thesis sought to explore if these same cytokines measured in the blood and spleen were increased in the rostral and caudal HF, locally to where DCX$^+$

density was reduced to the respective control group. In chapters 2+3 gene expression of IL-1β, IL-6 and TNF-α gene expression was measured across the HF in birds with severe vs minimal KBF and poor vs good physical body condition. In the data presented in chapter 3, there was no difference in gene expression of all genes measured across the HF. This was excluding IL-10 expression which was increased in the rostral HF of birds with severe KBF. It remains unclear why birds with severe fractures would have greater expression of anti-inflammatory cytokines as part of the immune response, although this could indicate a previous inflammatory presence which will be discussed later. In addition to no spleen changes as discussed above, there is no indication in a change of inflammatory cytokine expression between birds with good and poor physical condition. This was again the case in chapter 3 between birds with minimal and severe KBF, where the severity which were scored on a scale of severity by x-ray (Rufener, Baur, et al., 2018) of the fracture did not influence expression in the HF.

Chapter 3 however did draw some limitations with sampling, as fractures were present amongst all birds proving it difficult to have an unaffected control group. Still, it has been shown that the more severe fractures lead to reduced DCX^+ density compared to minimal fractures (Armstrong et al., 2020). This meant it could have been likely to see an effect of fracture on inflammatory cytokines in the HF where DCX^+ is reduced. In addition, KBF have already been demonstrated to influence gene expression of inflammatory cytokine IL-1β in hens (Wei et al., 2019), although the study in question measured brain tissue and not hippocampal tissue and made a comparison between present and absent fractures.

Due to the limitations within chapter 3, the next step was to run a correlation analysis between the actual fracture severity score for each individual bird with the respective gene expression. Consistently there was no correlation between the severity of the fracture and gene expression, suggesting the more severe a fracture is there is still no change in expression.

As the birds used for this study were obtained from a partially-commercial farm, all birds had acquired fractures at different times determined by 11 consecutive x-ray images (Armstrong et al., 2020). Therefore, as a final step, chapter 3 ran a further correlation analysis between gene expression and the time each bird’s fracture was first and last captured. These results as outlined in chapter 3 indicate a positive correlation between birds who acquired fractures for the first time closer to sampling had increased gene expression of IL-1β and decreased IL-10. This finding points toward an initial increase in IL-1β expression in the HF when a fracture is younger. However, birds who had presented fractures in the earlier x-rays which were longer lasting or even showing signs of healing by the point of the final x-ray had higher IL-6 gene expression in the rostral HF only. Together these results indicate it might not be the severity of the fracture which influences the inflammatory cytokine gene expression in the HF, it could be the fracture age where cytokine expression might increase during the early stages. This opens up the question of why birds with severe KBF have increased IL-10 gene expression in the rostral HF with no changes in inflammatory cytokines. It is likely that the expression of these cytokines work in conjunction with one another. IL-1β has been referred to as a primary cytokine which induces production of IL-6, both of which are involved with the inflammatory phase of bone remodelling (Maruyama et al., 2020), which could explain why IL-1β is increased in the HF of birds whose fractures were newer at the time of sampling followed by increased IL-6 in older fractures.

6.4 Microglia and associated markers in the HF during chronic stress

Microglia are the brain's resident macrophage and have roles within the brain to maintain homeostasis by removal of cellular debris and dying cells through phagocytosis. Microglia often do this without producing inflammation in their ramified state. However, upon brain injury microglia undergo morphological changes into their ameboid phenotype and produce inflammation (Gemma & Bachstetter, 2013). Chapters 2 and 3 took the first steps into investigating microglia by measuring CSFR-1, however there was no changes in expression between groups or any relationship with DCX+ density. Not only that, CSFR-1 is a marker of microglia activation which doesn't differ between phenotypes. It was clear that gene expression of CSFR-1 did not differ between birds with severe and minimal fractures, then again with good and poor physical condition. To understand if microglia are involved with chronic stress under inflammatory circumstances, for example in their ameboid phenotype further investigation was required. Therefore chapter 4 carried out a morphological analysis by counting IBA-1^+ cellular density of three morphological types of microglia in birds with KBF.

Chapter 4 took the birds with minimal and severe KBF where DCX$^+$ density in the HF was reduced in the later (Armstrong et al., 2020). This study predicted there would be an increase of inflammation producing ameboid microglia in the birds with severe fractures in comparison to minimal fractures, which was not the case. There was no difference in the three morphological subtypes counted: ameboid, ramified and rod between fracture groups. However, there was an overall increase in ameboid microglia compared to ramified and rod irrespective of fracture severity.

Ameboid microglia are producers of inflammatory cytokines including IL-1β, IL-6 and TGF-β, none of which were increased when measuring gene expression in the HF in chapter 3. As ameboid microglia density did not differ between minimal or severe KBF status similarly to how gene expression of the inflammatory cytokines did not, it again draws towards the limitations within this study as there were no birds with absent fractures to include as a control group. Nonetheless, as overall density of ameboid microglia was increased compared to other morphologies across all birds, it is unclear if this is driven by the presence of a fracture rather than the severity. This is unlike the negative effect severity has on DCX$^+$ density in the HF.

The lack of an appropriate control in chapters 3 and 4 was a major limitation in the interpretation of these results and therefore provided the rationale for chapter 5 which used UCMS to control the duration and severity of stress, whilst ensuring a negative control. If KBF leads to chronic stress which could lead to an increase in the presence of ameboid microglia, this could trigger activation of the inflammasome which contributes to inflammation via caspase-1 activation (S. Li et al., 2022; Karaffová et al., 2020). As a next step chapter 5 for the first-time measured gene expression of several markers of the inflammasome, including NLRP3 and caspase-1 which when activated lead to expression of IL-1β (S. Li et al., 2022).

NLRP3 is a multiprotein complex which is activated by damage associated molecular patterns (DAMPs) and is vital for regulation of the immune system (S. Li et al., 2022). Microglia have been associated with the NLRP3 inflammasome in a depression mouse model, which led to activation of neurotoxic astrocytes and neuronal dysregulation (S. Li et al., 2022). If the findings from chapter 4 imply the presence ameboid microglia is greater in birds with KBF compared to without, this could lead to activation of the NLRP3 inflammasome. However, where an appropriate negative control was included in the UCMS experiment in chapter 5, there was no increase in NLRP3 gene expression in the UCMS group. Neither was there an increase in caspase-1 and inducible nitric oxide synthase (iNOS). These findings alongside the findings in chapters 2 and 3 which point toward no evidence of inflammatory cytokine involvement. However, as discussed in chapter 3 birds who had acquired KBF closer to sampling had higher gene expression of IL-1β in the HF which is known to be secreted as part of the microglial NLPR3 inflammasome which ultimately leads to astrocyte activation (S. Li et al., 2022). So whilst this thesis did not find evidence of microglia morphology changes or changes in gene expression of microglial activation marker CSFR-1, it did find evidence to suggest activated astrocytes which are activated by microglia at an earlier stage. As in chapter 3 where IL-1β was increased in newer fractures, collectively these findings suggest detection of the later stages of inflammation in the HF during chronic stress.

6.5 The involvement of astrocytes and activation of the complement pathway during Unpredictable Chronic Mild Stress

UCMS has previously been shown to reduce neuronal plasticity in the HF of hens, by reduced DCX^+ density in the rostral and caudal HF (Gualtieri et al., 2019). There is currently no clear understanding of the physiological mechanisms which cause reduced neuronal plasticity consequently. This thesis has throughout sought to investigate if inflammation is involved, which there has so far been no evidence to suggest this is the case.

In addition to measuring gene expression of NLRP3, chapter 5 took a different approach beyond microglia and investigated the potential role of Astrocytes. In the mammalian literature, microglia have been shown to induce a neurotoxic type of astrocyte via the NLPR3 inflammasome in a model of depression (Luarte et al., 2017). Chapter 5 first measured gene expression of astrocyte marker glial fibrillary acidic protein (GFAP) which expression was increased in the caudal HF of birds with UCMS treatment. It appears that chronic stress in this case leads to an increase in caudal GFAP expression, however, the interpretation of this can only be limited. For example, it cannot be determined if this is a general increase of astrocyte cell numbers or more gene expression due to morphological changes. Chronic stress has been associated with morphological changes of various cell types in the brain, including astrocytes and microglia (Luarte et al., 2017). GFAP has been used in various studies to investigate astrocytes during chronic stress. One study in mice report 5 weeks of psychological stress led to a 25% reduction in the somatic volume of hippocampal astrocytes (Kwon et al., 2008). On the contrary, studies using the chronic restraint model found increased GFAP positive cell numbers and protein expression in the hippocampus of mice (Jang et al., 2008). In addition to increased GFAP expression in the caudal HF, UCMS led to increased gene expression of C3 across both the rostral and caudal HF. Astrocytes are large producers of complement and have been associated with the stress response, but not in birds. Therefore, the results in chapter 5 point towards an astrocyte driven activation of the complement pathway in the HF during UCMS. However, one downside of measuring gene expression in the HF is the inability to determine if astrocytes are responsible for this increase. This is especially as GFAP was increased across both rostral and caudal whereas C3 was only increased in the caudal HF.

In addition to UCMS, chapter 5 included two rearing types within the study: Those reared with dark brooders (DB) and without (conventional). Although the increase of GFAP gene expression occurred in both rearing types with UCMS treatment, rearing did influence C3 expression. UCMS did lead to increased C3 gene expression in comparison to the control group of both rearing types. However, it appears that birds with DB rearing have higher C3 gene expression compared to conventional birds independently of UCMS. Therefore, it could be the case that when birds are reared as DB's, they do not exhibit the same level of C3 gene expression increase as conventional birds do, but still experience increased GFAP. If chronic stress does lead to activation of the complement pathway in the HF which could be associated with reduced neuronal plasticity, it is interesting that when reared as DB C3 levels are at a comparable level to conventional UCMS. Craven (2024), who measured DCX gene expression in these same birds, found DCX expression is increased in the caudal but not rostral HF of DB birds with UCMS. It appears that the DB birds do not experience the same increase of C3 and reduced DCX gene expression as conventionally reared birds do, suggesting they do not respond to chronic stress in similar ways.

When birds are reared with DB, this is an early life intervention to provide some level of maternal care such as warmth and shelter the in the absence of the mother. These results could indicate that DB leads to increased stress resilience in later life that conventional birds do not experience.

6.6 Final thoughts and future directions

This thesis utilized three scenarios where birds who had experienced chronic stress had reduced neuronal plasticity than their respective control groups. In chapter 5 where birds had received UCMS treatment as a chronic stressor, there was increased GFAP gene expression in the caudal than rostral HF and increased C3 gene expression across the whole HF. As there was no increase in gene expression of any of the cytokines measured in the blood, spleen and HF alongside no changes in microglia morphological types or cell numbers between conditions, excluding IL-1β gene expression which was increased in birds with more recently acquired fractures. This suggests chronic stress leads to an astrocyte driven activation of the complement pathway in the HF which could involve the secretion of inflammatory cytokine IL-1β in a time dependent manner.

The findings of this thesis remain limited due to experimental reasons including measuring cell density of microglia in the absence of a controlled chronic stressor such as UCMS and measuring gene expression rather than protein levels. Therefore, it is important for the future direction of this research to take those limitations into account. For example, when birds had keel bone fractures whether that was minimal or severe there was increased hippocampal IBA-1^{+} ameboid microglia opposed to rod and ramified (Gualtieri et al., 2019). However, because there were no birds without a fracture it is not clear if those birds would too have more ameboid vs rod and ramified microglia. It is not clear if this would be expected in the HF of all birds in general, or what the relative abundance of different microglia morphologies typically is. In addition, when there was an appropriate control during the UCMS experiment in chapter 5, there was increased gene expression of C3 and GFAP. As microglia are involved with astrocyte activation via the NLRP3 inflammasome, it would be interesting to see if UCMS birds had increased ameboid microglia similarly to the KBF birds. Unlike the study in question, chapter 5 did not find increased NLPR3 gene expression suggesting this increase is not due to microglia driven activation of the inflammasome. Ideally this study would have included further microglia morphology analysis using IBA-1 where UCMS was able to provide an appropriate control which chapter 4 lacked. However, it was not possible to obtain further samples for histological analysis or brain tissue throughout UCMS. It would be interesting to see if microglia morphologies and cell numbers differed throughout the UCMS protocol. Future work would also include further investigation into astrocytes at the cellular level including cellular density and morphology, rather than gene expression alone would be a useful tool in determining the role of astrocytes during UCMS.

The results in chapter 5 which suggest UCMS leads to astrocyte driven activation of the complement pathway in the HF for the first time provide some evidence of the involvement of the immune system. However, because this study measured gene expression it cannot be concluded that astrocytes are responsible for activation of the complement pathway. As previously mentioned, future studies would benefit from going beyond measuring gene expression and focusing on cell numbers and morphology rather than gene expression. This is also the case for C3 where quantification of protein levels beyond the HF for example in the blood could provide more insight into the involvement and source of complement. Future experimental design should consider the early stages of chronic stress by taking samples beyond blood, including hippocampal tissue. This would enable greater measurement of cytokines in the blood and hippocampus, alongside the astrocyte and microglia markers measured in chapters 4 and 5. By doing so, it could be explored if there is an increase of inflammatory cytokines in the early stages of chronic stress and how long this persists for and if this is followed by increased astrocytes and related markers including C3.

Nonetheless, if increased C3 gene expression in the HF is an indication of chronic stress this could be a useful biomarker. This is especially as when birds with early life interventions such as DB rearing do not experience increased C3, this suggests C3 is sensitive to stress resilience.

One final point to address is the method this thesis used to determine gene expression in groups by using a ratio of inflammatory cytokine directly with anti-inflammatory cytokine IL-10. Chapters 2, 3 and 5 all used the same approach which included calculating the Zi of the log value of the inflammatory cytokines and IL-10 separately, then taking a direct ratio of inflammatory to IL-10. This was performed without prior normalisation of the inflammatory cytokine to the reference gene LBR, which was used throughout this entire study. All future publications of this work and future study would benefit from normalising the gene of interest with the reference gene to remove biological variation, before performing a ratio with IL-10 to determine pro-inflammatory to anti-inflammatory expression.

To conclude, this thesis aimed to explore the involvement of the immune system during chronic stress of hens from three studies where reduced hippocampal plasticity was observed as a consequence (Gualtieri et al., 2019; Armstrong et al., 2022, 2020). There is evidence to suggest that during chronic stress, there is the presence of inflammation within the HF, which potentially begins with microglia. Although there was no difference of the microglia morphologies measured between KBF severity status, all birds had increased ameboid microglia relative to rod and ramified. However, birds who acquired fractures closer to the time the samples were obtained had increased gene expression of IL-1β, whereas birds who had acquired fractures earlier had increased IL-6 gene expression. In addition, birds who received UCMS treatment had increased gene expression of GFAP in the caudal HF and C3 across the rostral and caudal HF in chapter 5. Ultimately, these findings suggest chronic stress leads to activation of the complement pathway which is potentially driven by astrocytes which are activated by microglia during the later stages of inflammation. As IL-1β was increased in birds who had newer KBF in chapter 3 which is a product of the microglial NLPR3 inflammasome (S. Li et al., 2022), there is reason to suggest microglia involvement. Moreover, birds who had DB rearing compared to conventional, did not experience the same increase of C3 gene expression during UCMS. This suggests DB rearing as an early life intervention could promote stress resilience later in life which measuring C3 expression in the HF could pose as a potential biomarker.

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