Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

Neal O. Fenner
BSc (Hons)
Doctor of Philosophy (PhD)
July 2012
Statement of Originality

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Name

./...../....

Date

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Acknowledgements

I would like to acknowledge the support of my supervisors, Dr Peter Hunt and Associate Professor Deborah Hodgson for their guidance and patience during the last couple of years. Without their constant support this thesis would not have made it to fruition. It's been hard but I can finally see light at the end of the tunnel.

Also I would like to acknowledge the support and friendship of all staff involved at the CSIRO in Armidale, especially Dr Nick Andronicos (a living Wikipedia) and Jody McNally for their assistance inside and outside of the laboratory.

To all the PhD students at Chiswick, thanks for your friendship and I hope all your aspirations are realised.

Lastly to my family, I don't know where I would be without you. You give my all the love and support a man needs and more. There's been dark times and it frightens me to think what would have become if you weren't around. You are my inspiration.
Table of Contents

CHAPTER 1: ..............................................................................................................................1

Literature Review

1.1 Aims
1.2 Background
  1.2.1 The sheep industry
1.3 The Immune Response to Gastrointestinal Nematodes
  1.3.1 The innate immune response
  1.3.2 From innate to acquired immune response
  1.3.3 The acquired immune response
1.4 The Hypothalamus-Pituitary-Adrenal Axis
  1.4.1 The hypothalamus
  1.4.2 The anterior pituitary gland
  1.4.3 The adrenal cortex
1.5 Perinatal Programming
1.6 Glucocorticoids
1.7 The Effects of Early Life Programming on the Immune Response
1.8 Implications of Study
1.9 References

CHAPTER 2: ..........................................................................................................................47

Differences in Immune Response to Ovalbumin between Lewis and Fischer Rat Strains

2.1 Introduction
  2.1.1 Molecular & behavioural differences between Lewis & Fischer rat strains
  2.1.2 Glucocorticoid regulation of the immune response
  2.1.3 Inflammation and the HPA axis
  2.1.4 Immune responses to ovalbumin
  2.1.5 Hypothesis

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
2.2 Materials & Methods

2.2.1 Animals
2.2.2 Experimental Design
2.2.3 Euthanasia, animal dissection and tissue preparation
2.2.4 Quantitative PCR
2.2.5 Radioimmunoassays
2.2.6 Total extractable lipids
2.2.7 Statistical Analysis

2.3 Results

2.3.1 Corticosterone response to model antigen immune stimulation
2.3.2 Gene expression changes in the prescapular lymph node
2.3.3 Gene expression changes in the hypothalamus
2.3.4 Gene Expression changes in the adrenal gland
2.3.5 Other physiological observations

2.4 Discussion

2.5 References

CHAPTER 3: Differences in the Primary Immune Response and Subsequent HPA Axis Response to Heligmosomoides bakerii between Lewis and Fischer Rat Strains

3.1 Introduction

3.1.1 Mouse immune responses to H. bakerii
3.1.2 Rat immune responses to H. bakerii
3.1.3 Rodent regulation of immune responses to gastrointestinal nematodes
3.1.4 Aims & Hypothesis

3.2 Materials & Methods

3.2.1 Animal trial, worm dosing and worm passage
3.2.2 Quantitative PCR
3.2.3 Corticosterone Radioimmunoassay
3.2.4 Histology
3.2.5 Statistical Analysis

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
3.3 Results

3.3.1 Hippocampus gene expression
3.3.2 Hypothalamus gene expression
3.3.3 Adrenal gland gene expression
3.3.4 Small intestine gene expression
3.3.5 Mesenteric lymph node gene expression
3.3.6 Spleen gene expression
3.3.7 Plasma corticosterone changes
3.3.8 Liver analysis
3.3.9 Body Temperature
3.3.10 Body Weight
3.3.11 Food Consumption
3.3.12 qPCR analysis of parasite DNA in small intestinal tissue
3.3.13 Faecal egg counts

3.4 Discussion

3.5 References

CHAPTER 4: .................................................................................................. 173

Co-culture of rat gastrointestinal cell lines with Heligmosomoides bakerii induces changes in gene expression: a model for gastrointestinal nematode infection in vitro

4.1 Introduction

4.1.1 The role of IL-33 and IL-25 in gastrointestinal immune responses
4.1.2 The role of basophils & dendritic cells
4.1.3 Cytokines: effects on gastrointestinal epithelial cells
4.1.4 Glucocorticoids: effects on gastrointestinal epithelial cells
4.1.6 Experiment design
4.1.7 Aims
4.1.8 Hypothesis

4.2 Materials & Methods

4.2.1 Cell Culture
4.2.2 DEX dose response
4.2.3 Response to parasitic gastrointestinal nematodes

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
4.3 Results

4.3.1 Effects of glucocorticoids on cell growth
4.3.2 Effects of glucocorticoids on basal cytokine expression
4.3.3 Gene expression changes in response to *H. bakerii* & the effects of glucocorticoids

4.4 Discussion

4.4.1 Effects of glucocorticoids on cell growth & basal gene expression
4.4.2 The co-culture of IEC6 cells & *H. bakerii*

4.5 References

4.6 Appendix

**CHAPTER 5:** Discussion & Conclusions

5.1 Experimental Overview
5.2 Conclusions
5.3 Closing Remarks

**CHAPTER 6:** Materials & Methods

6.1 Euthanising of Rat, Tissue Isolation and Tissue Storage
   6.1.1 Rat Euthanisation and Tissue Isolation & Storage
   6.1.2 White Blood Cell Isolation & Storage
   6.1.3 Spleenocyte Isolation

6.2 RNA Extraction & Storage

6.3 Quantitative PCR
   6.3.1 Two Step qPCR
   6.3.2 Primer Design
   6.3.3 Gene Fragment Cloning & Sequencing Using Transformed E.coli Cells
   6.3.4 Assay Optimisation

6.4 Cortisol RadioImmunoAssays
6.4.1 Principle of Assay
6.4.2 Rat RadioImmunoAssay
6.4.3 Sheep RadioImmunoAssay

6.5 Histology
6.5.1 Tissue Histology
6.5.2 Immunohistochemistry

6.6 Lipid Extraction from Animal Tissue

6.7 Heligmosomoides bakerii
6.7.1 Passaging & Culturing H. bakerii
6.7.2 L3 Exsheathing

6.8 Standard Cell Growing Procedure
6.8.1 Standard Passaging Procedure

6.9 Reagents

CHAPTER 7: ...........................................................................................................255

Supplementary Experiments

CHAPTER 8: ...........................................................................................................280

Poster Presentations
Abbreviations

ACTH: adrenocorticotrophic hormone
AF: Accessory Transcription Factor
AO: Alum + Ovalbumin
AP: Activator Protein
APC: Antigen Presenting Cell
AVP: Arginine Vasopressin

BZ: Benzimidazole
cAMP: Cyclic Adenosine Monophosphate
CD: Cluster of Differentiation
CLIP: Corticotrophin Like Immediate Lobe Protein
COX: Cyclooxygenase
CREB: Cyclic AMP Response Element Binding Protein
C/EMP: CCAAT Enhancer Binding Protein
CRH: Corticotrophin Releasing Hormone
CRH1R: Type 1 CRH Receptor
CTL: C Type Lectins

DC: Dendritic Cell
DEX: Dexamethosone

ELAM: Endothelial Leukocyte Adhesion Molecule

FECs: Faecal Egg Counts
FO: Freunds + Ovalbumin

GATA: GATA Binding Protein
GR: Glucocorticoid Receptor
GRE: Glucocorticoid Response Element
GRU: Glucocorticoid Response Unit

HPA: Hypothalamic-Pituitary-Adrenal

ICAM: Intracellular Cell Adhesion Molecule
Ig: Immunoglobulin
IFN: Interferon
IL-1: Interleukin 1
IL-1R: Interleukin 1 Receptor
IRAK: IL-1 Receptor Associated Kinase

LPS: Lipopolysaccharide

MAPK: Mitogen Activated Protein Kinase
MC2R: Melanocortin 2 Receptor
MHC: Major Histocompatibility Complex
ML: Macrocyclic lactones
MLN: Mesenteric Lymph Node
MMP: Multipotent Precursor Cells
mRNA: Messenger Ribonucleic Acid
MSH: Melanocyte Stimulating Hormone
NPY: Neuropeptide Y
NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells
NADPH: Nicotinamide Adenine Dinucleotide Phosphate H
N-POC: N Terminal Proopiomelanocortin
NO: Nitric Oxide
P450SCC: P450 Side Chain Cleavage
PAMPs: Pathogen Associated Molecular Patterns
PC: Prohormone Cleavage
PEPCK: Phosphoenol Pyruvate Carboxykinase
PKA: Protein Kinase A
PLN: Prescapular Lymph Node
POMC: Proopiomelanocortin
rER: Rough Endoplasmic Reticulum
SCP: Sterol Carrier Protein
SEA: Schistosome Soluble Egg Antigen
SF-1: Steroidogenic Factor 1
StAR: Steroid Acute Regulatory Protein
STAT4: Signal Transducer & Activator of Transcription
RELM: Resistin Like Molecule
TCR: T Cell Receptor
TH1: T Helper 1
TH2: T Helper 2
TLR: Toll Like Receptor
TNF: Tumour Necrosis Factor
TRE: Tissue Plasminogen Activator Response Element
TREG: T Regulatory Cells
VCAM: Vascular Cell Adhesion Molecule

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Abstract

The aim of this study was to establish a model for examining the role of the neuroendocrine system in regulating the immune response of the host to gastrointestinal nematodes. Through this model the role of the neuroendocrine system in responding to parasitism and regulating the T Helper 2 (TH2) response was investigated. Specifically, Fischer and Lewis rats were used to discover whether differing, but physiological glucocorticoid levels altered the response to parasites. In addition, a cell culture model was used to examine the interaction between parasites and dexamethasone in epithelial cell gene expression. Results showed that whilst gene expression patterns differed between the two rat strains, the key responses in the immune and gastrointestinal systems did not differ. Both rat strains mounted an effective response to parasites, blocking the lifecycle before adult, egg laying nematodes could develop. In contrast, the addition of high levels of dexamethasone in a cell-culture model changed cellular gene expression responses to parasitism significantly. Results suggest that immune responses can remain effective despite marked changes in physiological glucocorticoid levels, but that artificial glucocorticoid administration can exceed a threshold above which significant immunosuppression can occur. Intriguingly, gene expression responses to parasitism were observed in brain tissues and adrenal glands providing a glimpse of the capacity for signals to be transmitted from the site of parasitism by the neuro-endocrine system.
1.1 Aims

The proposed aim of this study is to establish a model for examining the role of the neuroendocrine system in regulating the immune response of the host to gastrointestinal nematodes. Through this model the role of the neuroendocrine system in regulating the T Helper 2 (TH2) response will be investigated. Specifically, the study will ask the question “Does hyper-glucocorticoid secretion promote a stronger TH2 response, effective in expelling gastrointestinal nematodes”? Analysis of the T cell phenotype, protein and mRNA levels of the type 1 and type 2 cytokines, as well as glucocorticoid concentration, and antibody titres will establish whether a TH1 or TH2 response has been generated and to what extent. The initial investigation will utilise a rat model due to availability of molecular tools in which to analyse the response. If this model generates data of interest, subsequent experimentation will involve sheep. Sheep are a key species which suffers from parasitism; however the lack of molecular tools available, long gestation interval and greater costs associated with sheep experimentation preclude the use of this species for initial experiments.
1.2 Background

1.2.1 The Australian Sheep Industry
Wool exports are worth over $3.3 billion to Australia’s annual economy, being the third largest agricultural export. Since 1807 Australia has been exporting wool overseas, firstly to England and now to China, Japan and Italy. As of 2006 Australia was the largest exporter of greasy wool accounting for 26% of the world’s greasy wool trade. From humble beginnings to the economic prosperity of the 1980’s the wool market has been a significant part of Australia’s Gross Domestic Product. However, the industry is facing an uncertain future with challenges from a changing world wool market, draught, climate change and anthelmintic resistance.

Australia: Built off the Sheep’s Back
Since the first fleet in 1788 sheep have been an integral part of Australia. Sheep have been used as both meat and wool in Australia, with the first sheep in Australia being primarily for meat. In 1807, the first wool bale was exported to England by John MacArthur. Over 80% of sheep in Australia are Merinos tracing blood-lines back to Spain, with the majority of the remainder being of Merino-crosses. Five main Merino strains exist in Australia, these being Peppin, Emperor, Spanish, South Australian and Saxon.

Wool production in Australia is an important part of the economy with over 50,000 wool growing properties, being the largest form of land use. Sheep numbers in Australia peaked in the 1980’s at 172 million head, however due to drought and failing markets this number had declined to 98 million head in 2004. As of 2003, Australia was the largest producer of greasy wool in the world. Wool exports account for 13% of the agricultural export and 2.4% of the total export, valuing wool exports at over $3.3 billion.

Figure 1.1: Top Wool Producing Countries.
Europe is the largest producer of greasy fleece wool in the world with a 26% share of the global production. Source: AWI (2007)
billion, third behind beef and wheat.

Due to selection pressures favouring characteristics, such as fleece weight, fibre diameter & staple strength, and carcass traits the rise of susceptibility of the Merino to infection by gastrointestinal nematodes has occurred. Gastrointestinal nematode infection is a significant problem facing the wool industry, with the cost estimated at $400 million annually, which is significantly higher than that of blowfly and lice [1]. In order to combat gastrointestinal nematode infection, anthelmintics (anti-helminth chemotherapies) have been extensively used in Australia. Parasite resistance to anthelmintics was reported in the 1960’s and is increasing in Australia and the world due to frequency of usage, low dosages, the use of persistent anthelmintic formulations, and environmental-treatment interactions [2]. In order to understand anthelmintic resistance, mode of action of anthelmintics and molecular mechanisms behind resistance needs to be addressed.

The Rise of Resistance and the Molecular Mechanisms of Anthelminitics

Predicting the development of resistance is difficult, as shown in the rapid development of Ivermectin resistance, seen in Haemonchus contortus, contrasting with resistance in Trichostrongylus colubriformis which is less common. However, Levamisole resistance is common in T. colubriformis, but uncommon in H. contortus [3]. The complexity of the parasite lifecycle is a significant factor in developing resistance. Parasites with simple lifecycles are more likely to develop resistance more rapidly than that of parasites with complex lifecycles [3]. Resistance to Benzimidazoles (BZ), Macro cyclic Lactones (ML) and Imidazothiazoles has been well documented in Australia [4, 5] and overseas [6-8]. Through interfering with β-tubulin, BZs are able to inhibit microtubule polymerisation. Mechanisms for BZ-resistance have been extensively studied. Through mutations of the β-tubulin gene, resistance occurred [9]. This was later shown to coincide with an amino acid substitution at position 200, with Tyrosine
replacing Phenylalanine (reviewed [6]). However, it is more likely that changes in population allele frequencies, instead of gene rearrangement, plays a major role in the development of resistance [6]. Various other studies have shown other less conclusive mechanisms involving P-glycoproteins, egg shell fluidity and a role for cholesterol (reviewed by [6]). The mechanism behind resistance to Imidazothiazoles is thought to involve nicotinic acetylcholinesterases receptors. Sangster & Gill [3] suggested that a reduction in the number of nicotinic acetylcholinesterases receptors or reduction in Imidazothiazole affinity to these receptors may be the molecular mechanism behind resistance. MLs affects parasites through paralysis and starvation. ML compounds act on glutamate-gated Cl⁻ ion channels possibly affecting muscles of the pharynx, therefore inhibiting feeding. Similarly, paralysis of somatic musculature may occur [3]. The mechanisms behind ML resistance has limited coverage in literature [3] however, Eng et al. [10] showed that Ivermectin selected for a single nucleotide polymorphism in Onchocerca volvulus, resulting in a three amino acid change in the H3 helix of β-tubulin. Resistance was also conferred to Caenorhabditis elegans having mutations in all three of the glutamate-gated Cl⁻ ion channel genes avr-14, avr-15 and glc-1. Interestingly, resistance was severely reduced if mutation occurred in only two of these genes [11]. Anthelmintics act through a number of molecular mechanisms to reduce parasite fitness. However, through selection these mechanisms can be bypassed, allowing for the rise of resistance into a population. It is evident that the rise in resistance is more likely to be a function of increasing allele frequency, than that of gene rearrangement in a population, and that lifecycle complexity can effect the evolution of resistance in a population [11, 12].
1.3 The Immune Response To Gastrointestinal Nematodes

The immune response to gastrointestinal nematodes presents a challenge to the immune system since these parasites are eukaryotic, multicellular organisms residing in the extracellular environment. Parasitic nematodes possess factors which modulate the host's immune response, and are capable of residing in dedicated niches within the host. These niches such as the abomasum and the small intestine come into contact with foreign antigen on a daily basis, therefore the host needs to be able to distinguish between harmless and potentially harmful antigens and mount an appropriate response. In order to mount an effective immune response against gastrointestinal nematodes, the host uses an arm of the immune system known as the humoral immune response, characterised by TH2 cells and type 2 cytokine synthesis [12].

1.3.1 The Innate Immune Response

The innate immune response is the first line of defence of the host in recognition of antigens. Although the innate immune response is seen as evolutionarily less developed than that of the acquired immune response it still plays a vital role in protecting the host against harmful pathogens.

Pro-Inflammatory Cytokines & the Generation of Inflammation

Cytokines are important regulators of the immune response. Their vast array of functions includes regulation of inflammation/anti-inflammation, cell growth and differentiation, resource allocation, activation of chemotaxis & cell adhesion, and stimulation of the Hypothalamus-Pituitary-Adrenal (HPA) axis. Cytokines are small proteins distinct from hormones in that they are released from many cell types especially during immunological challenge. Interleukin-1β (IL-1β) has been shown to stimulate noradrenaline in the paraventricular nucleus of the hypothalamus and median eminence, and may be the mechanism behind cytokine stimulation of the HPA axis and parallels adrenocorticotropic hormone (ACTH) release [13]. Similarly, IL-1β has been shown to increase the release of Neuropeptide Y (NPY), noradrenaline and

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*
*N.O. FENNER (2012)*
adrenaline from chromaffin cells [14]. IL-1β, Tumour Necrosis Factor α (TNFα) and IL-6 are important mediators involved in the generation of inflammation and are collectively known as pro-inflammatory cytokines and are important in generating the inflammatory response. Pro-inflammatory cytokines are produced by macrophages, dendritic cells, mast cells, monocytes and various other leukocytes. IL-1β and TNFα stimulate the activation of the Nuclear Factor-kappa B (NFκB), an important transcription factor in the transcription of genes involved in the inflammatory immune response such as cell adhesion molecules, anti-apoptotic factors, cytokines, transcription regulators and growth factors [15].

Pro-inflammatory cytokines act through activation of transcription factors necessary for the inflammatory response. One such example is the activation of the NFκB transcription factor in promoting cyclooxygenase-2 (COX-2) transcription. The generation of inflammatory-related prostaglandins is reliant on the COX-2 enzyme and prostaglandins are central to the inflammatory response [16]. The rate-limiting step in prostaglandin synthesis relies on the activities of COX-2, where COX-2 catalyses the conversion of arachidonic acid to eicosanoids, which are converted to prostaglandins via the actions of prostaglandin isomerases [16]. Regulation of COX-2 mRNA levels is dictated, at both the transcriptional and post-transcriptional level, by mitogen activated protein kinase (MAPK). MAPK regulates COX-2 mRNA levels through the activation of cyclic-AMP response element binding protein (CREB), NFκB, and CCAAT-enhancer binding protein (C/EBP). COX-2 is also regulated at the enzymatic level by nitric oxide (NO), where NO increases the catalytic activity of the COX-2 enzyme. This occurs due to the formation of peroxynitrite anions from the reaction between NO and superoxide ions, enhancing COX-2 activity [16, 17].

An important aspect of inflammation is the migration of effector cells such as neutrophils, mast cells and eosinophils to the site of infection. Acute inflammation involves the migration of neutrophils, resulting in the characteristic swelling of the infected area. The recruitment of these cells requires the
expression of cell adhesion molecules and chemokines. Adhesion molecules expressed on vascular endothelial cells near the site of infection ‘capture’ effector cells, such as neutrophils which are passing through systemic circulation. These captured cells then penetrate the vascular endothelium entering the inflamed tissue and migrating towards a chemotactic gradient generated by infected and/or damaged cells. Induction of IL-1β or Interferon γ (IFNγ) synthesis plays a crucial role in the initiation of the inflammatory response inducing chemokines [18]. Similarly, cytokines are also involved in cell adhesion molecule expression. TNFα is involved in the upregulation of Intercellular Cell Adhesion Molecule (ICAM-1), Vascular Cell Adhesion Molecule (VCAM-1), and Endothelial-Leukocyte Adhesion Molecule (ELAM-1) in severe malaria cases [19]. TNFα has also been shown to upregulate ICAM-1 in cerebral malaria [20]. Therefore, cytokines are important in both cell adhesion molecule expression and chemokine synthesis, demonstrating the central role of cytokines during the inflammation response.

1.3.2 From Innate to Acquired Immune Response

*Inflammation Helps Recruit T Cells*

Inflammation results in the migration of leukocytes to the site of infection, with T cell antigen recognition being conducted in lymphoid tissues associated with the tissue of infection. Recruitment of monocytes to the site of infection allows for antigen presentation to naïve T Helper cells initiating differentiation into either TH1 or TH2 subsets. Cytokine-induced expression of cell adhesion molecules and chemokines plays a crucial role in lymphocyte homing [21]. Therefore, inflammation-related cytokines are essential for the differentiation of lymphocytes through their role in recruitment of these cells to lymphoid tissues in preparation for antigen presentation.
A Novel Role for Basophils

The leukocytes mast cells, eosinophils and basophils are important mediators in allergenic reactions. Through the release of mediators and cytokines these effector cells are able to activate, mediate and stimulate the immune response. Although basophils represent less than 1% of the total circulating granulocytes population, recent research has suggested that basophils are crucial in antigen presentation in generating the TH2 [22-30]. Basophils are crucial in establishing chronic allergenic inflammation mediated through Immunoglobulin E (IgE) antibodies [23, 31]. Importantly, these results demonstrated the protective importance of basophils in response to helminth infection [23]. Basophils are able to produce IL-4 and IL-13 through two main routes being stimulation by Ag/IgE complex or through reception of IL-33 or IL-18 in the presence of IL-3 [22]. IL-3 being important for the priming process of basophils [32]. Basophils have recently been found to act as antigen presenting cells (APCs) possessing Major Histo-compatability Complex II (MHCII) and co-stimulatory molecules Cluster of Differentiation 40 (CD40), CD80, and CD86 [22, 24-26]. Although dendritic cells are able to act as APCs in TH2 responses they are not exclusively required rather antigen presenting basophils are able to exclusively induce a TH2 response and are needed despite the presence of dendritic cells [27, 29].

1.3.3 The Acquired Immune Response

The acquired immune response is an arm of the immune system which through initial contact with a given antigen is able to learn an immune response of increase efficiency.

The Role of T Helper Cells: The TH1/TH2 Response

Processing and presentation of antigen by APCs is a crucial step in the differentiation of naïve T-cells expressing the CD4+ marker. These cells differentiate into either TH1 or TH2 subsets. Processed antigen peptides presented via MHC proteins are essential for CD4+ cell differentiation. Priming
of naïve CD4+ cells has been suggested to be a qualitative function of the affinity strength of Toll-Like Receptors (TLR) which recognise foreign antigens [28]. Immature Dendritic Cells (DCs) residing in peripheral tissue are activated through interaction with Pathogen-Associated Molecular Patterns (PAMPs), pro-inflammatory cytokines or T-Cell derived cytokines. C-type lectins (CTL) may play a role in the internalisation of some antigens, such as those present in Schistosome soluble egg antigen (SEA) [30]. Van Liempt [30] also demonstrated that internalisation of antigen by DC cells could be achieved without TLRs, rather relying upon CTL and the mannose receptor in SEA antigen processing and presentation by MHC II.

The mature DC achieves APC status through migration to draining lymph nodes, whilst upregulating ICAM-1, CD40, CD80 and CD86 in readiness for T cell stimulatory cytokine expression, as well as significant upregulation and stabilisation of MHC molecules [24, 25].

IL-12 is required for differentiation into TH1-type cells and IL-4 (and possibly IL-13) is required for TH2-type cells. IL-12 dependant maturation of primed CD4+ cells results in differentiation into the TH1 cell subsets and the expression of type 1 cytokines, whilst IL-4 dependant maturation of primed CD4+ cells results in the differentiation into the TH2 subset and the expression of type 2 cytokines. Differentiation and maturation of the primed CD4+ T cell relies on the availability of pro-TH1 cytokines such as IL-12, IL-18 and possibly IL-23 [28]. Binding of IL-12 by IL-12 receptors (IL-12R) results in the activation of the Signal Transducers and Activators of Transcription 4 (STAT4) pathway. Also, IL-18 acts in synergy with IL-12 to assist in differentiation. IL-18 activates the IL-1 Receptor Associated Kinase (IRAK) and NK-κB pathway, when combined with the IL-12 activated STAT4 pathway results in the upregulation of IFNγ.

The differentiated TH1 cell is able to further produce IFNγ, regulating TH1 differentiation and enhancement of macrophage-mediated killing. IFNγ is also important in the down regulation of IgA expression and has been described as a hallmark cytokine for a TH1 response [33, 34]. IFNγ is essential for TH1 commitment and its activity also induces IL-12 receptor expression and inhibits

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
the TH2 pathway [35]. IL-12 and IL-18 may play a critical role in TH1 cell-mediated susceptibility to parasitic nematodes, characterised by IFNγ secretion, through the subsequent downregulation of IL-13 [34]. A host genotype, typified through increased IL-12 or IL-18 expression may be causative in susceptibility of the host to parasitic nematodes [34]. The main function of IFNγ is to stimulate the activities of macrophages, and suggesting INFγ activated macrophage activity plays a role in the cell-mediated immune response [36].

The TH2 response is a crucial element in the effective immune response resulting in expulsion of parasitic nematodes [37-44]. A strong TH2 immune response has been shown to correlate with resistance to worm burden and reinfection and typically involves (but not exclusively) mastocytosis, eosinophilia, B cells proliferation and antibodies production of IgE and IgG1, as well as TH2 dependant type 2 cytokine production (reviewed [45]).

Priming of CD4+ T cells occurs on contact with APCs, through MHC and T cell receptor (TCR) interaction. Expression of IL-4 follows resulting in autocrine release of IL-4 and self stimulation which results in the activation of STAT-6. STAT-6 commits the cell to expression of type 2 cytokines through the differentiation of the naïve T cell into TH2 cells. Similarly, GATA binding protein-3 (GATA-3) has been shown to play a crucial role in Type 2 cytokine expression in TH2 cells [46].

*Downregulation of the Immune Response*

Downregulation of the immune response is important for two responses. Firstly, over-stimulation or over activation of the immune response can cause autoimmunological damage to the host. This damage may be caused by tissue damage from histamine release, increased cyclooygenase activity, swelling and heat, and tissue necrosis caused by inflammation. This would be deleterious to the host as resources would be used in repairing tissue in place of reproduction or fighting various other infections. Secondly, over-stimulation of the immune response would require a great deal of resources which may result in muscle wasting, reduced substrates needed for cell division such as amino acids and
nucleic acids, and reduced overall host fitness.

**Downregulation of Inflammation and the Innate Immune Response**

Inflammation, as an innate response to infection, is crucial in the recruitment of immune cells to the site of infection. These cells, once at the site of infection produce mediators, which enhance the inflammatory response, such as synthesis of pro-inflammatory cytokines by monocytes. These cytokines play a crucial role in the recruitment and activation of lymphocytes, therefore establishing the acquired immune response. These pro-inflammatory cytokines are monitored by the hypothalamus, promoting catecholamine synthesis and activating the HPA axis. Activation of the HPA axis results in glucocorticoid synthesis which, combined with catecholamines, control inflammation. However, glucocorticoids and catecholamines also have been shown to favour the TH2 phenotype through actively suppressing the TH1 phenotype. Therefore, activation of the neuroendocrine system may have two functions; controlling inflammation and promotion of humoural immunity.

Catecholamines

Catecholamines have also been shown to have anti-inflammatory properties. Noradrenaline has been shown to inhibit the synthesis of IL-12, whilst adrenaline enhances IL-10 synthesis in Lipopolysaccharide (LPS) stimulated human whole blood cultures mediated through β-adrenoceptors [53]. Importantly, β2-adrenoceptors are expressed on TH1 cells and not TH2 cells [53, 55], thereby allowing catecholamines to influence TH1 cells exclusively. Therefore, both glucocorticoids and catecholamines are able to downregulate the inflammatory response, subsequently shifting the immune response from a cellular TH1 response to a humoural TH2 response.

**The TH2 Response in Action: Response to Heligmosomoides polygyrus**

*H. polygyrus* is a natural parasite of mice but not of rats. However, *H. polygyrus* is able to infect rats causing significant pathology even though the parasite life...
cycle is retarded to an extent where parasites are trapped in the submucosal stage. Many parasites induce strong TH2 immune responses in hosts and *H. polygyrus* is no exception. Ingestion of the larvae by mice and rats introduces the parasite to host gastrointestinal system. From here the larvae migrate to the host's gastrointestinal tract where they target the duodenum and the proximal jejunum [56, 57]. Maturation occurs in submucosal tissue of the duodenum and the proximal jejunum where the mature worms emerge from tissue cysts into the lumen to reproduce, releasing viable eggs into the contents of the gastrointestinal tract. These eggs are released to the environment via the host faeces. The larvae hatching in the faeces migrate to the surface and await consumption by another host to complete the life cycle. Mice of different strains show varying levels of susceptibility and resistance. High responder strains include SJL, SWR, mid-range responders include BALB/c, NIH, B10G, and DBA, and low responders include CBA & C3H C57bl/10 [57]. High responder strains expell larvae at an increased rate and effectiveness, had higher IgG1 than that of low responder mouse strains demonstrating the importance of a strong TH2 immune response [57]. Similarly, effective parasite immune responses can be reversed through CD4+ depletion resulting in reduced IgE response [58]. Antibody producing B cells are therefore an important component in the immune response to *H. polygyrus* however B cells are also essential for helping establish and drive the TH2 immune response through secretion of cytokines [59].

*H. bakerii* has also been shown to cause an inflammatory response in rats but is unable to reproduce [60-64]. Whilst able to penetrate the rat gastrointestinal mucosal tissue, an intense inflammatory response is generated causing extensive connective tissue generation and rendering the larvae unable to re-emerge as seen in the mouse [60, 61]. The inflammatory response seen in the rat can be abated through corticosterone treatment, which results in a response similar to that of the mouse where the larvae re-emerge and are able to reproduce [60]. Therefore, glucocorticoids are important in the susceptibility of rats to *H. bakerii* infection. Inflammation in this instance is crucial for resistance,
at least initially, causing entrapment of the larvae in the gut mucosa though connective tissue genesis.

The TH2 Response in Action: Schistosomiasis & the Response to the Egg

Schistosomiasis

Schistosomiasis is a disease caused by trematodes of the *Schistosoma* genus, infecting more than 200 million people worldwide [65]. Mortality due to *Schistosoma mansoni* in sub-Saharan Africa is estimated at 280,000 per annum [65]. Schistosomal infection is also of importance in South America. Asian schistosomiasis is caused by *Schistosoma japonica*. As with other parasites, Schistosome infection induces a strong TH2 immune response. The immune response is influenced by a number of factors including host genetics, infection intensity, *in utero* sensitisation and co-infection [65]. Acute Schistosomiasis is characterised by detectable levels of TNFα in plasma as well as production of pro-inflammatory cytokines by peripheral blood mononuclear cells. This usually coincides with low egg production by the parasite from initial infection until 6 weeks post infection. However, during the egg production period of infection, downregulation of pro-inflammatory cytokines occurs, shifting the immune response from a predominant TH1 to a TH2 response. It can therefore be seen that egg production causes a shift in T cell populations, demonstrating the properties of SEA that makes it suitable as a model TH2 antigen. SEA is secreted from tissue-encapsulated eggs and is most commonly extracted for use from *S. mansoni* and less often, *S. japonicum*.

Response to the Schistosome Egg & and generation of a Granuloma

Response to the Schistosome egg, located within the host’s liver, is observed as a TH2 response, through the secretion of SEA by encapsulated eggs. Generation of a granuloma in egg-containing tissue results in damage to that tissue as an observable symptom in the host during chronic infection.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

*N.O. FENNER (2012)*
Granulomas form as a result of the host immune response in extracellular parasitic eggs generating a TH2 response. This response manifests into a granuloma due to the accumulation of macrophages and eosinophils, as well as synthesis of collagen, directed by CD4+ cells. As the eggs are degraded, the granuloma reduces leaving fibrotic plaques [66, 67].

SEA is internalised by DCs through CTL and presented via MHC II, whilst inhibiting production of IL-12, TNFα, IL-6 and IL-10 [30]. Therefore, CTL may play a crucial role in DC antigen presentation to T cells resulting in the establishment of the TH2 response. Stimulation with SEA shows increased regulation of the CD4+CD25+ response through IL-10 activity. Experimentally induced asthma demonstrated impaired regulation of mucosal TH2 responses. However, regulation was restored on treatment with SEA, inducing IL-10 but not IL-4 secretion [68]. This result suggests that allergenic diseases, such as asthma, are a result of deregulation of the T cell response. Therefore, IL-10 is shown to be important in the regulation of T cell immune responses. In vitro stimulation of peripheral blood mononuclear cells from individuals with high and low burdens of *S. mansoni* eggs demonstrated an increase in INFγ secretions in the low compared to the high group. However, the high group had increased levels of IL-10, whilst IL-13 remained unchanged between groups [69]. This therefore suggests that INFγ and IL-10 play a role in the immune response to SEA demonstrating the role of TH2 cells in SEA induced immune responses.
1.4 The Hypothalamus-Pituitary-Adrenal Axis

The HPA axis is a central part of the response to stress. The hypothalamus is the link between the nervous system and the endocrine system. Corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) secreting hormones are important in the stress response and originate from the hypothalamus. Other important neuron secreted peptides include dopamine, Gonadotropin-releasing hormone, melatonin, somatostatin, and thyrotropin-releasing hormone. The secretion of CRH is crucial for the endocrine response to stress. On secretion CRH binds to receptors on the corticotrophic cells of the anterior pituitary. This results in the synthesis and cleavage of pro-opiomelanocortin (POMC) to produce adrenocorticotrophic hormone (ACTH). Secretion of ACTH into systemic circulation allows for binding to the melanocortin receptor (MC2R) of the adrenal cortex. This process initiates a signalling cascade resulting in the increased production of cortisol. Cortisol plays an important role in the response to stress through breakdown of glycogen, lipids and proteins, as well as anti-inflammatory effects. A negative feedback mechanism exists where regulation of cortisol synthesis is controlled by hippocampus influenced regulation of the hypothalamus.

1.4.1 The Hypothalamus

The hypothalamus is a small region of the brain, located below the thymus in the diencephalon region and is responsible for the monitoring of the organisms internal milieu. Neurons from the hypothalamus project into the median eminence between the hypothalamus and the pituitary gland and are responsible for regulating the activity of the pituitary. Examples of hypothalamic originating peptides important in cortisol synthesis include CRH and AVP. Other important hypothalamic peptides include dopamine, gonadotropin-releasing hormone, melatonin, somatostatin, and thyrotropin-releasing hormone. Originating in the paraventricular nucleus of the hypothalamus are the CRH and AVP parvicellular neurons. Parvicellular neurons terminate at the median eminence secreting CRH and AVP into the blood supply of the anterior pituitary.
Primary AVP secreting parvicellular neurons and secondary magnicellular
eurons secrete AVP in concert with CRH into the median eminence where the
systemic circulation brings corticotrophic cells into contact with these hormones
to enhance ACTH synthesis and secretion [70]. Alterations in CRH levels also
account for altered growth, immune and reproductive activities [71].
Importantly, the paraventricular nucleus is within the blood-brain barrier
however parvicellular neurons project into the median eminence, a region
where the blood-brain barrier is reduced. Possible routes by which cytokines
can enter the brain are at areas of the blood brain barrier where there is
increased permeability such as that found at the median eminence, or through
specific transport systems [72]. It therefore may be possible that IL-1β and other
‘CRH releasing’ cytokines bind to the CRH and AVP neurons at the median
eminence, allowing for release of CRH and AVP into the systemic blood supply
via the hypophyseal portal system [73]. John & Buckingham [72] also suggest
that ‘CRH releasing’ cytokines may target the paraventricular nucleus of the
hypothalamus through association with perivascular cells of the blood brain
barrier causing the release of mediators such as eicosanoids. Induction of CRH
and AVP secretion also follows diffusion of eicosanoids into the paraventricular
nucleus where Buckingham et al. [74], demonstrated that IL-1β, IL-6 and TNFα
induced secretion of CRH mediated locally by activities of eicosanoids.
However, Rook [75] suggests that IL-1β may bind to receptors in the periphery,
causing activation of sensory and vagal afferent nerves allowing the location of
inflammation to be identified. It is possible that both these mechanisms work in
synergy, allowing an integrated approach to immune monitoring.
The hypothalamus also influences the activity of the locus coeruleus, through
vagal and sensory afferent nerves located at the site of inflammation [70, 75,
76]. Through the release of ‘CRH releasing’ cytokines such as IL-1β, IL-6 and
TNFα at the site of inflammation [70, 73], sensory nerves convey neurotransmitters back to the locus coeruleus resulting in secretion of
noradrenaline via efferent sympathetic neurons at the site of inflammation [77].
This causes a similar response to glucocorticoids, with promotion of a TH2

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
cytokine profile as well as various anti-inflammatory responses induced through the reception of noradrenaline by β-adrenergic receptors of given immune cells [77]. Efferent vagus nerves also interact with the site of inflammation through release of Acetylcholine (Ach), which mediates inflammation through N-Ach receptors on macrophages [77]. Secretion of CRH into the locus coeruleus is also critical for the activation of the release of corticosterone and the generation of stress-related behaviours [78]. Parvicellular CRH neurons terminate at close proximity to catecholamine and tyrosine hydroxylase neurons, expressing CRH receptors [78]. Tyrosine hydroxylase enzymes are the rate limiting step in the synthesis of catecholamines [79]. It’s also possible that communication may be bi-lateral with repressive efferent nerves down-regulating aspects of the paraventricular nuclei [70].

The role of the hypothalamus during the immune response involves responses mediated through the HPA axis, the arcuate nucleus and the locus coeruleus. During inflammation the hypothalamus detects increased levels of pro-inflammatory cytokines through the systemic circulation and via activation of the nervous system. Detection of inflammation results in the secretion of CRH into the median eminence, promoting ACTH secretion and cortisol synthesis. CRH is also secreted into the arcuate nucleus which increases levels of pain-relieving peptides, such as β-endorphin, derived from POMC cleavage. Secretion of CRH into the locus coeruleus involves activation of the hypothalamus via signals conveyed from the site of inflammation. Detection of pro-inflammatory cytokines and other inflammatory mediators by afferent nerves relay signals to the locus coeruleus promoting efferent nerve secretion at the inflammatory site. This results in directed site-specific release of anti-inflammatory mediators such as noradrenaline by sympathetic efferent nerves resulting in downregulation of inflammation. With the responses of both the arcuate nucleus and the locus coeruleus the central mediating factor is CRH derived from the hypothalamus.
The classical HPA axis involves the monitoring of the internal milieu, where CRH and AVP are secreted when the hypothalamus detects stress. CRH binds to CRH receptors of corticotrophic cells of the anterior pituitary. This increases ACTH synthesis. ACTH binds to MC2R of the adrenal gland. This increases cortisol (corticosterone) synthesis which is secreted into the systemic circulation. Cortisol downregulates inflammation and frees resources to assist in the stress response. Levels of cortisol are monitored by the hippocampus (and to a lesser extend the hypothalamus, the anterior pituitary and the adrenal gland) and this regulates cortisol synthesis in a negative feedback loop. CRH secretion into the locus coeruleus and arcuate nucleus increases noradrenalin, Ach and β-endorphin, which is secreted at the site of inflammation to downregulate inflammation.

In chronic inflammation, the level of AVP expression is increased whereas CRH is maintained at a relatively steady rate [80]. The exception is during the early stages of inflammation where CRH expression is increased (reviewed by [81]. Therefore, AVP plays a crucial role in the dynamics of the HPA axis response to inflammation, however it is unclear whether chronic pain or pain related cytokines are the contributing factors to differing HPA responses [80]. Chowdrey et al. [82] showed that AVP mRNA expression is increased, while CRH mRNA is unchanged in the parvicellular neurons of the paraventricular nucleus of the hypothalamus.
hypothalamus, whilst ACTH expression was increased in the pituitary of arthritic PVG rats. Therefore, this suggests that AVP up regulation acts to enhance the activity of CRH in the regulation of ACTH expression of the pituitary. Systemic CRH may also play a role at the site of inflammation. Radio-labelled CRH was shown to bind to peripheral blood lymphocytes and monocytes as well as splenocytes (reviewed by [81]). Similarly, CRH expression has been found in both the sensory and sympathetic neurons, which suggests a role for CRH at the site of inflammation, both at a cellular level and a neurological level.

Figure 1.3: The Interaction between the HPA axis & the Gastrointestinal Tract. Parvicellular neurons originating from the paraventricular nucleus of the hypothalamus secrete CRH, regulating ACTH synthesis in the anterior pituitary, β-endorphin synthesis in the arcuate nucleus and noradrenaline & Ach synthesis in the locus coeruleus. Activation of the locus coeruleus through pro-inflammatory cytokine monitoring initiates CRH secretion into the locus coeruleus, initiating noradrenaline secretion at the site of inflammation.

Regulation of CRH Synthesis

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
CRH secretion promotes the transcription of the POMC gene within the arcuate nucleus. The POMC gene contains the gene products for ACTH, alpha-, beta-, and gamma-Melanocyte-stimulating hormone (MSH), beta- and gamma-Lipotrophin (LPH), Corticotrophin-like intermediate lobe peptide (CLIP), N-terminal Pro-Opiomelanocortin (N-POC) and β-endorphin [83]. The gene product undergoes post-translational proteolytic cleavage via the activity of prohormone convertases [84], where the resulting protein product is tissue specific [85, 86]. The resulting protein product, β-Endorphin, cleaved within the arcuate nucleus, influences the paraventricular nucleus through CRH down regulation [87], as well as pain control at other areas of the brain [70]. The arcuate nucleus region of the hypothalamus is responsible for the majority of β-endorphin synthesis [88], with β-endorphin synthesising neurons projecting widely in the brain [89]. Therefore, there are two negative influences on the paraventricular nucleus, the cortisol-mediated feedback mechanism and the cleaved POMC protein products loop.

1.4.2 The Anterior Pituitary Gland

The anterior pituitary plays an intermediate role in the activity of the HPA axis. CRH reception by corticotrophic cells of the pituitary initiates transcription of the POMC gene, which undergoes post-translational processing to produce the ACTH peptide which is released into the systemic circulation. Interestingly, the POMC gene encodes for a number of various proteins of differing activities. Post-transcriptional cleavage of the POMC peptide yields various products dependant on tissue. The enzyme responsible for the cleavage is prohormone convertases 1 and 2 (PC1 & PC2). The POMC gene is large consisting of 7,665bp and containing three exons. Of these, exon 1 is untranslated, whilst exon 2 encodes the signalling peptide as well as the first N-terminus amino acids. The majority of the translated gene is contained in exon 3 [90]. The signalling peptide is of importance as it allows for the translocation of the nascent peptide into the rough Endoplasmic Reticulum (rER), where the signalling peptide is rapidly cleaved. The newly formed peptide is then

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?  
N.O. FENNER (2012)
transported within the intracellular traffic to the Golgi Bodies and subsequently secreted. It is here, during transportation in the intracellular traffic that the peptide is cleaved to form the maturated ACTH protein. The cleavage enzyme PC1 located in the corticotrophic cells cleaves the POMC peptide into the bioactive ACTH protein, whereas, in the arcuate nucleus PC2 allows for a different cleavage subset, including β-Endorphin, to be produced. The corticotrophic cells of the anterior pituitary express only the PC1, whilst the arcuate nucleus expresses both. This allows for second round cleavage, resulting in a differing subset of peptides [90]. Tissue specific cleavage of the POMC polypeptide is achieved through the use of different populations of similar cleavage enzymes which recognise differing cleavage sites.

CRH is the most potent and physiologically important stimulator of POMC gene transcription, where CRH binds to type 1 CRH receptors (CRH1-R) of the corticotrophic cells of the anterior pituitary [91, 92]. Stress activates the hypothalamus resulting in an increase in CRH expression and release into the pituitary portal system by CRH parvicellular neurons [93]. There is however little correlation between the abundance of CRH1-R and the transcriptional responsiveness of the corticotrophic cells, which suggests that CRH1-R numbers is not important in pituitary responsiveness since a low number of CRH1-R can elicit an effective response to most stressors [71]. The number of CRH1-R is not dependant on CRH1-R mRNA levels, which suggests a negative feedback role for cortisol at the level of translation [71]. POMC transcription is controlled by the Activator Protein-1 (AP-1) transcription factor, which is comprised of either fos-jun heterodimers or jun-jun homodimers, which bind to the Tissue Plasminogen Activator Response Elements (TRE) of the POMC gene promoter [74, 94]. CRH reception induces c-fos transcription, which has been shown to be stimulatory for POMC gene expression and is a major component of AP-1 bound to the TRE promoter region [94]. Similarly, Boutillier et al. [94] found that c-fos independent POMC stimulatory pathways exist showing that deletion of the major AP-1 binding site reduced c-fos mediated POMC transcription but failed to suppress CRH mediated POMC expression. It

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
has been shown that CRH stimulated POMC transcription involving the AP-1 transcription factor is a phasic process, whereby the composition of AP-1 changes overtime. Aguilera et al. [71] showed that c-fos plays a major role during the early stages of CRH induced POMC transcription with an increased abundance of c-Fos\Jun-B dimers, while the later stage is characterised by Fra-2\Jun-B dimers.

CRH modulates POMC expression and therefore the cleavage of ACTH through the binding activities of CRH1-R. Importantly, AVP increases the level of CRH1-R mRNA and therefore accounts for the up regulation of ACTH during stress [71]. CRH1-R is found predominately in the pituitary as a plasma membrane receptor of the corticotrophic cells, while CRH2-R is found in discrete areas of the brain importantly the arcuate nucleus [71]. ACTH secretion by the corticotrophic cells is dependant upon both the level of CRH as well as the number of CRH1-R molecules present. The degree of sensitivity of these receptors is an important factor in the level of ACTH secretion [71]. Korosi et al. [95] showed that chronically elevated CRH levels were shown to markedly elevate CRH2-R while inhibiting CRH1-R in various parts of the brain. Therefore, chronically elevated CRH levels may also inhibit CRH1-R expression at the corticotrophin cellular level.
Neuronal signals originating from the paraventricular nucleus of the hypothalamus secrete CRH and AVP into the median eminence, promoting ACTH synthesis at the pituitary. Secretion of ACTH from the pituitary promotes cortisol synthesis and secretion into the systemic circulation. Signals from the paraventricular nucleus also initiate POMC synthesis and β-endorphin synthesis in the arcuate nucleus of the hypothalamus. Production of β-endorphin interacts with the immune response and promotes analgesia. Signals from the paraventricular nucleus, through CRH secretion into the locus coeruleus promotes noradrenalin and acetylcholine synthesis. Cortisol, noradrenalin and acetylcholine all contribute to downregulation of inflammation. The blue lines represent neuronal pathways, while the red lines represent systemic blood supply pathway.

1.4.3 The Adrenal Cortex

The binding of CRH to CRH1-R promotes the synthesis and cleavage of the POMC peptide to produce ACTH through the activity of PC1. ACTH is then transported via the systemic blood supply to the adrenal glands where binding to its receptor, the MC2R, causes a signal transduction pathway that leads to the activation of the cortisol synthesis pathway [96].

The rate of cortisol & corticosterone synthesis is determined by the binding of ACTH to the MC2R, a receptor on the surface of adrenal cells within the zona fasciculate (cortisol)/reticularis (corticosterone) of the adrenal cortex.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
The rate-limiting step of steroidogenesis is controlled by the cytochrome P450 Side-Chain Cleavage (P450SCC) enzyme. P450SCC is encoded by the gene CYP11A1 and has steroid hydroxylase activity important in the initial step of steroidogenesis, the conversion of cholesterol to pregnenolone [97]. The level of CYP11A1 gene expression is determined by the reception of ACTH, activating adenylate cyclase and generating a Cyclic Adenosine Monophosphate (cAMP) mediated signalling pathway [98]. Activated Protein Kinase A (PKA) initiates steroidogenesis via a number of routes [96], including the activation of cholesterol ester hydrolase which generates free cholesterol from cellular cholesterol ester stores [97], activation of the transcription factors Steroidogenic Factor-1 (SF-1), AP1\CREB and Sp1, which assemble on the promoter region of the CYP11A1 gene [98].
Figure 1.5: Reception of ACTH to the Melancortin-2 Receptor Results in Activation of PKA. PKA is involved in the phosphorylation of transcription factors involved in the transcription of gene which mediate Cortisol synthesis. Reception of ACTH to the Melancortin-2 Receptor initiates a G-coupled protein cascade which results in the activation of adenylate cyclase. Adenylate cyclase then converts ATP to cAMP which binds to and releases PKA from its repressor chaperon. PKA then enters the nucleus and activates transcription factors involved in the transcription of genes involved in Cortisol synthesis.

A conformational change in the promoter region then increases RNA polymerase II association and transcription, and the resulting protein product, cytochrome P450SCC, located on the mitochondrial inner membrane [99]. The reduction of cholesterol to pregnenolone is a step-wise process involving an electron transport chain. The electron transport chain located on the inner mitochondrial membrane involves the steroid hydroxylase activity of P450_{SCC}, combined with the co-factors ferredoxin and ferredoxin reductase and electron donation of Nicotinamide Adenine Dinucleotide Phosphate H (NADPH) [99]. The

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure 1.6: Cholesterol Reduction Electron Transport Chain. A pair of electrons are donated to the FAD group of Ferrodoxin reductase. These electrons are then passed to ferrodoxin. Ferrodoxin transports the electrons to P450\textsubscript{SCC}, where the electrons move to the haem centre. The haem centre is now charged to allow for reduction of cholesterol.

The rate of steroidogenesis is acutely modulated by transport of free cholesterol to P450\textsubscript{SCC}, achieved through the activity of the carrier protein steroid acute regulatory protein (StAR). StAR is essential for the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. There are a number of models which describe the activity of StAR in membrane translocation [100] including the hydrophobic protein core, the lipid bridge and StAR acting as a cholesterol binding protein.

Acute activation of steroidogenesis is governed primarily by the ability to transport cholesterol to P450\textsubscript{SCC}, achieved through the activity of StAR. Chronic activation of steroidogenesis, however, is governed by transcription of the CYP11A1 resulting in an increased abundance of the P450\textsubscript{SCC} cytochrome [97, 101, 102]. Similarly, chronic ACTH reception involves cAMP pathway, where the CREB mechanism is not involved [96, 97]. It is hypothesised that during the innate response in sheep there would be primarily little difference in the

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
abundance of CYP11A1 mRNA compared to the naive control, however, during a chronic infection it would be expected that there would be an increase in CYP11A1 mRNA due to the chronic response initiated by an increased activity of the HPA axis.

Sterol carrier protein 2 (SCP2) may also be a sterol transfer protein, transferring cholesterol from the cytoplasm to the mitochondrial outer membrane [103]. Once cholesterol has been reduced to pregnenolone it is transported to the smooth endoplasmic reticulum where it undergoes hydroxylation to form 11-deoxycortisol. 11-deoxycortisol is then transported back to the mitochondria where it is converted to cortisol by P450 11β-hydroxylase (also CYP11B1), located on the inner mitochondrial membrane [104]. In sheep, the single enzyme CYP11B1 is responsible for the production of glucocorticoids as well as mineralocorticoids and is located in the mitochondria. Cortisol synthesis is 100-1000 fold higher than that of aldosterone due to both transcriptional regulation and catalytic activity [105].
1.5 Glucocorticoids

Glucocorticoids are derivatives of cholesterol synthesised in the adrenal gland. Glucocorticoids such as cortisol and corticosterone are naturally synthesised steroids important in the stress response. The name glucocorticoid is derived from the fact that these steroids are involved in the regulation of glucose. The synthesis pathway of glucocorticoids is a tightly regulated process, and is the endpoint of HPA axis activation.

Anti-Inflammatory Effects

Cortisol is the main glucocorticoid hormone produced by the adrenal glands of humans and is the major biological endpoint of activation of the HPA axis. Furthermore, cortisol also negatively feeds back into the HPA axis to regulate its own production. Cortisol mediated downregulation of the inflammatory response is essential in the regulation of the immune response and in preventing auto-immune damage. Cortisol, which is synthesised by the adrenal glands through a series of enzymatic reactions carried out by the CYP family of cytochrome enzymes, is released into systemic circulation where it binds to the cytosolic GR. On binding, the heat shock protein hsp90 is released allowing for the receptor complex to translocate into the nucleus, acting as a transcription factor to enhance transcription of genes with GRE contained with their promoter region. Downregulation of inflammation is achieved through the transcription of IκB, a protein inhibitor of NFκB with a GRE in the promoter. Inhibition of NFκB downregulates pro-inflammatory cytokines and therefore the inflammatory response [47-49]. Monitoring of the immune response by the hypothalamus, through detection of pro-inflammatory cytokines, allows cortisol synthesis to respond to inflammation. The GR also implements biological actions independently of the GRE by physically interacting with other transcription factors such as AP-1 and NF-κB [49, 115, 116]. Cortisol mediated anti-inflammation also involves the downregulation of NOS, Phospholipidase A2 and Cyclooxygenase 2, and adhesion molecules [117, 118]. Cortisol also upregulates endonucleases, which are involved in the apoptosis of lymphocytes.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?  
N.O. FENNER (2012)
and eosinophil [118].

Figure 1.7: Mechanism for Anti-Inflammation.  
**Cortisol Excess:** After binding of free Cortisol, the GR monomer dimerises and enters the nucleus, where it binds to the GRE initiating IκB expression, which inhibits NF-κB, downregulating type 1 cytokine expression.  
**Cortisol Shortage:** TNF binds the TNF receptor activating IκB kinase. IκB kinase tags IκB for ubiquination, allowing NFκB to enter the nucleus and promote type 1 cytokine expression.

The annexin family of proteins are found in all eukaryotic cells, where annexin1 may be involved in anti-inflammatory effects through inhibition of phospholipase A2. This results in the inhibition of eicosanoid synthesis, blocking the migration of leukocytes and inducing the apoptosis of inflammatory cells [119]. Similarly, annexin1 also inhibits the expression of cyclooxygenase and nitric oxide synthase through increased IL-10 [119]. This shows that glucocorticoids can elicit ant-inflammatory effects both directly through physically inhibiting inflammation transcription factors and by transcribing genes which have anti-inflammatory roles within the cell.  
Immediate effects take place within the cell far more rapidly than that seen by

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*  
*N.O. FENNER (2012)*
transcription related cortisol effects, suggesting that cortisol is involved in non-transcriptional processes in the cell. These effects include changes to actin structures, neuronal membranes and transmembrane currents [120]. Mineralocorticoids in mammalian systems are able to activate Ca\(^{2+}\) mediated second massager pathways which have been suggestive of membrane bound GR [121].

**Glucocorticoid-mediated Resource Allocation**

Glucocorticoids are synthesised as a response to stress. This response involves downregulation of the inflammatory response to prevent auto-immunological damage caused by an unregulated immune response. Glucocorticoids are also able to mediate resource allocation in response to stress. By mediating the allocation of resources, glucocorticoids are able to supply cells with resources needed to restore homeostasis and recover from the stressor. Examples of this include the freeing of glucose from the liver through gluconeogenesis, break down of skeletal muscle, increasing blood pressure, breakdown of lipids and proteins, mobilisation of amino acids and ketone bodies, production of hypotonic urine and increased effectiveness of catecholamines.

Increases in levels of glucose in the blood are mediated through the increased transcription of the gluconeogenesis rate limiting enzyme, Phosphenolpyruvate carboxykinase (PEPCK) through the activities of GR and glucagon-mediated cAMP [122]. Binding of GR and accessory transcription factors (AF) AF1, AF2, and AF3 to the glucocorticoid response unit (GRU) promotes the transcription of PEPCK [122]. Increased levels of transcription leads to gluconeogenesis as observed during fasting. During immunological stress higher levels of glucocorticoids leads to the freeing of glucose through PEPCK-mediated gluconeogenesis. Free glucose in the blood is important for supplying tissues and cells with energy in order too counter-act the stressor. For example, increased levels of free glucose caused by an increase in glucocorticoids in response to immunological stress allows for an increase in energy to be

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
available for the immune response. The skeletal muscle of an organism represents a large emergency store of amino acids in the form of myosin and actin filaments and is a significant consumer of cellular energy. Glucocorticoids may induce skeletal muscle atrophy to release amino acids for use in protein synthesis or as energy stores. Inhibition of skeletal muscles to utilise glucose in times of stress may also allow for further glucose to be available for other tissues such as brain and the immune response [122]. Glucocorticoid induced muscle atrophy is associated with increased levels of myostatin, activation of ubiquitin pathways and primarily affects fast-twitch muscle fibres (type II), including catabolic effects on myosin heavy chains [123, 124]. Myostatin is an inhibitory growth factor, inhibiting muscle growth. The presence of glutamate inhibits glucocorticoid mediated muscle atrophy by downregulating myostatin expression. Glutamine synthetase is crucial in the metabolism of glutamate, condensing glutamate and ammonia to form glutamine. Glucocorticoids upregulate glutamine synthetase mRNA and protein levels in skeletal muscle C2C12 cell lines. When cultured in the absence of glutamine protein levels were increased but not mRNA levels. This suggests that glutamine is needed for translation of glutamine synthetase in the presence of glucocorticoids [125]. Therefore, the upregulation of myostatin inhibits muscle growth, whilst upregulation of glutamine synthetase allows removal of glutamate from the muscle.

The Effects of Glucocorticoids on T Helper Cell Differentiation

Glucocorticoids are described as anti-inflammatory and immunosuppressive, however these conclusions have been concluded due to the use of pharmacological dosages, which are not consistent with biological levels [43]. Recent research suggests that glucocorticoids may be immunoenhancive, downregulating pro-inflammatory cytokines and the TH1 phenotype, while promoting the type 2 cytokine profile and the TH2 phenotype [50-54]. One mechanism suggested involves the inhibition of IL-12 production by corticosteroids in APCs, resulting in downregulation of IFNγ and upregulation of
IL-4 synthesis by CD4⁺ lymphocytes [51]. This effect is hypothesised to be a result of reduction of the blocking effects of IL-12 on IL-4 synthesis by glucocorticoids [51-53]. The synthesis of anti-inflammatory cytokine IL-10 is unaffected by glucocorticoids in monocytes and is upregulated in lymphocytes (reviewed by [53]).

**Molecular Differences between Cortisol & Corticosterone**

Cortisol is the primary glucocorticoid synthesised by the adrenal glands in humans. Corticosterone, on the other hand, is the primary glucocorticoid synthesised by rodents. Corticosterone is produced in humans but is an intermediate precursor to aldosterone. Corticosterone is produced by the conversion of deoxycorticosterone by CYP11B2 (also aldosterone synthase). Corticosterone is then converted to 18-O-corticosterone then aldosterone by CYP11B2. Deoxycorticosterone can also be converted to cortisol through firstly conversion to deoxycortisol by P45017a, and then cortisol by P450c11.

Therefore, the main difference between cortisol and corticosterone in humans is that cortisol is synthesised as the primary glucocorticoid, while corticosterone is an intermediate in the synthesis of aldosterone. In rodents, corticosterone is the main glucocorticoid, while aldosterone is also synthesised to regulate sodium ion excretion.

![Figure 1.8: The Molecular Structures of Cortisol (left) & Corticosterone (right).](image: www.icgeb.trieste.it)
1.6 Perinatal Programming

In 1994, Barker et al. [106-108] published findings suggesting a link between foetal under-nutrition and disease in later life. It was suggested that in utero under-nutrition of the foetus resulted in a susceptibility to diseases such as cardiovascular disease, non insulin-dependant diabetes mellitus, and the insulin resistance syndrome. Similarly, Rook et al. [109] described the interactions with transient harmless antigens during the perinatal stage may be critical in programming our immunoregulatory circuits. Deficiency in perinatal exposure to antigens may result in the development of autoimmune diseases such as asthma, inflammatory bowel disease and type 1 diabetes. Rook et al. [109] further suggests that the rise in autoimmune diseases in the developed countries may be due to a lack of exposure to micro-organisms in early life, resulting in inefficiencies in immunoregulation. Research is also emerging of the effects of maternal stress on the development of the foetus and subsequent outcomes in later life. Prenatal stress is shown to cause behavioural changes, brain alterations and affect immune function. Neonatal stress exposure to the neonatal has also shown to effect brain and immune development. This chapter will examine the effects of pre-and neonatal exposure to stress, in particular immunological stress caused by endotoxin exposure.

Perinatal Programming of the Immune System

The perinatal period of development typically refers to the time around birth, both before & after. The World Health Organisation defines the perinatal period as ‘the period commencing at 22 completed weeks of gestation (154 days) when birth weight is normally at least 500g and ends 7 completed days after birth’ in humans. Therefore, perinatal programming refers to changes in development initiated in the period immediately before or after birth. Perinatal programming in this instance refers to changes in neuronal development caused by both prenatal and/or neonatal exposure to stress. Prenatal exposure to stress is usually in the form of maternal stress interfering with foetal development [110]. Neonatal exposure to stress can include immunological
stressors such as infection [111, 112], psychological stressors such as maternal deprivation [113], or nutritional stressors such as food deprivation [114]. Possibly, in nature all three types of stressors may occur concurrently. Perinatal programming of the neuroendocrine system can affect the immunology and psychology of the individual.

Maternal stress results in programming of the foetus prenatal neuroendocrine system. Experimentally, this stress is applied to the pregnant mother through the acoustic startle method, involving exposure to a loud sound after a period of relative quietness. Acoustic startle is an advantageous method for applying stress to the expectant mother as there is no direct contact with the foetus. Therefore, the change in the development of the foetus’s neuroendocrine system is due to the stressful maternal perception of the acoustic startle.
1.7 The Effects of Early Life Programming on the Immune Response

It is well established that exposure to early life stressors during the perinatal period of development can affect the development of the neuroendocrine system, notably the HPA axis. Recent research also suggests that glucocorticoids, once thought as being immunosuppressive, may in fact play a crucial role in T cell differentiation, the acquired immune response and prevention of autoimmunological damage to the host. The question therefore arises does early life programming of the HPA axis affect the immune response and to what extent can this be either prevented or exploited?

Glucocorticoids & the Immune Response

Although pharmacological levels of glucocorticoids induce immunosuppression, evidence is emerging of a more subtle role for glucocorticoids at a biological level. Glucocorticoids are now known to suppress type 1 cytokine expression, such as IL-12, INFγ, IFNα, & TNFα in TH1 cells and APCs. Glucocorticoids however have the opposite effect in TH2 cells, upregulating IL-4, IL-10 and IL-13. Taken together this suggests that glucocorticoids may be involved in not only the suppression of inflammation but the shift from cellular to humoral mediated immunity [52]. Elenkov [52] suggests that glucocorticoids downregulate the TH1 response in order to prevent autoimmunological damage caused by chronic inflammation. Elenkov [52] further suggests that activation of the stress response (glucocorticoid synthesis) due to chronic stressor exposure may lead to an increased susceptibility to infection. As described above glucocorticoids promote anti-inflammation and T helper shift due to the inhibition of NFkB, a transcription factor important in the expression of type 1 cytokines. By inhibiting type 1 cytokines and subsequently the TH1 response the balance is shifted allowing for the TH2 phenotype to dominate. The TH2 phenotype is involved in the immune response to parasites such as gastrointestinal nematodes, as well as regulating antibody production and other humoral processes.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Figure 1.9: The Role of the HPA Axis in TH Regulation. Activation of the HPA axis causes increase cortisol synthesis. This promotes the TH2 phenotype leading to worm expulsion.

Hypothesised Effects of Early Life Programming on the Immune Response to Gastrointestinal Nematodes

Early life programming, caused by perinatal exposure to stress, affects the development of the HPA axis and therefore glucocorticoid responses to stress in later life. Since glucocorticoids have been involved in the TH1/TH2 ratio and the TH2 phenotype is important for parasite immune responses then it is logical that early life programming of the HPA axis must affect the hosts ability to mount an efficient immune response.
1.8 Implications of Study

An understanding of the role of the neuroendocrine system in regulating the response to gastrointestinal nematodes is crucial in the development of alternative strategies to anthelmintic chemotherapies. The role of the neuroendocrine system during stress is well documented. However, limited studies have investigated the role of the neuroendocrine system under immunological stress. Even less research has investigated its role during immunological stress caused by parasite infection. Increased knowledge about the role or roles of the neuroendocrine system during parasite mediated immunological stress may allow for the development of drugs to target key areas of the neuroendocrine system, or allow for selective breeding to select for individuals possessing favourable genotypes, allowing for a more efficient immune response. Although this study is conducted with an agricultural slant, various results may have implications in human medicine. These may include the effects of perinatal stress on the susceptibility of the host to fight off parasitic challenges. Therapeutics may be developed to minimise/optimise the levels of perinatal stress exposure, therefore allowing an alternative to anthelmintic usage.

Alternatives to Reliance on Anthelmintics

Parasites have over time developed resistance to many of the commonly used anthelmintics used in agriculture. The small ruminant industry is reliant on the anti-parasitic properties. The main focus of the outcomes of this study is to develop an alternative to anthelmintic usage due to the development of increasing anthelmintic resistance in small ruminants of agricultural importance such as sheep and goats. Through an understanding of the role of the neuroendocrine system during the immune response alternatives to anthelmintics can be developed. These alternatives may include chemotherapies designed to optimise the role of the neuroendocrine system, or selective breeding for optimal neuroendocrine performance.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Animal Welfare

The implications of this study may also apply to animal welfare. Stress caused to the mother during the later stage of foetal development has been shown to affect the foetus in later life. Therefore, from an animal welfare aspect, reducing stress during pregnancy of livestock may help to prevent deleterious developmental effects from occurring in the developing foetus, therefore increasing fitness and this may possibly relate to immune function. Similarly, neonatal exposure to stress especially endotoxin has been shown to affect the development of the rat neuroendocrine system. Therefore, be preventing bacterial infections during pregnancy may affect the immune response of the neonate in later life. Understanding the role of the neuroendocrine system in regulating the immune response to gastrointestinal nematodes may assist in establishing better animal husbandry practices to prevent abnormal neuroendocrine development.
1.9 Hypothesis

The degree to which the HPA axis responds to immunological stress, in conjunction with HPA axis basal activity, affects the shape of the immune response in terms of T helper cell differentiation.
1.9 References

24. Sokol, C.L., et al., Basophils function as antigen-presenting cells for an allergen-induced T


92. Lundblad, J.R. and J.L. Roberts, Regulation of proopiomelanocortin gene expression in

---

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)


Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?  

N.O. FENNER (2012)  

44
CHAPTER 2: Differences in Immune Response to Ovalbumin between Lewis and Fischer Rat Strains

2.1 Introduction
The HPA axis is an important regulator of homeostasis. Changes in homeostasis prompt increased activity of the HPA axis resulting in glucocorticoid synthesis, as well as activation of downstream neural and endocrine responses [1, 2]. It is widely believed that glucocorticoids have anti-inflammatory, as well as immunosuppressive properties. However, recent research suggests that these conclusions were based on pharmacological and not biological glucocorticoid concentrations [3-10]. Glucocorticoids may be involved in T-Helper cell differentiation, with physiological-scale elevations in plasma glucocorticoid levels promoting the TH2 phenotype [3, 4, 9-14] through suppression of TH1 differentiation. Therefore, although glucocorticoids show anti-inflammatory properties, the biological outcome of an increase in glucocorticoid levels can stimulate TH2 immune responses critical for immune-mediated removal of some parasites [15]. The work described in this chapter aimed to examine the immune response of rats with differing HPA axis phenotypes to assess the role of the HPA axis in regulating the immune response to model antigens. This experiment aimed to examine the way in which the Fischer and Lewis rat strains differ in response to immunological stress. These stressors have been selected to polarise the immune response to stimulate the TH1 and TH2 mediated T cell responses. These responses will be assessed by measuring type 1 and type 2 cytokine gene expression, corticosterone, body temperature and liver fat content. This experiment is designed to provide evidence of how the HPA axis may influence the immune system by utilising a simple antigen-adjuvants to provide the immune stressor, eliminating other influences from parasites such as the role of e/s products and mechanical damage. Evidence of HPA axis involvement will provide a proof of concept, allowing for the examination of further complex...
immunological stressors such as gastrointestinal nematode infections.

2.1.1 Molecular & Behavioural differences between Lewis & Fischer rat strains

The Fischer and Lewis rat strains are widely used in psychological research and are used commonly as a model for the study of HPA axis/immune interactions [16, 17]. The Fischer strain is known to have a higher basal level of corticosterone, which results in a phenotype which is resistant to inflammation. When exposed to streptococcal cell wall peptidoglycan polysaccharide (SCW), Lewis rats exhibit a markedly reduced response in terms of corticosterone response compared to the response of Fischer rats [17]. The reduced ability to respond to stressors in the Lewis strain is a result of reduced activity in the corticosterone synthesis feedback loop, where CRH is reduced in synthesis and secretion [2, 18]. This difference in HPA axis activity is also paired with decreased type 2 GR activation in neuronal and immune tissues [19]. Studies examining the immune response of Lewis and Fischer rats to Mycoplasma pulmonis showed that although both strains were susceptible to infection, the Lewis strain failed to recover and developed worsening physiological symptoms, whilst Fischer eventually cleared the infection after an infective period. One mechanism behind this was the increase in all classes of lymphocytes in Lewis, whilst only selective increases in certain lymphocyte classes in Fischer. Crucially antibody levels in both strains were not significantly different [20]. The phenotypic effects of reduced HPA axis activity results in inflammation, restraint and autoimmunity susceptibility [16]. Physiological differences include reduced corticosterone levels, reduced type 2 GR activity, and reduced CRH synthesis and secretion, and differing classes of lymphocyte proliferation. Fischer rats also demonstrate elevated corticosterone levels for a greater length of time as a result of stressors such as LPS, nicotine and novel stimulation [21] and are more stress-sensitive and less addiction prone [22]. In contrast, the Lewis strain has low levels of plasma corticosterone and demonstrates an inflammation susceptible phenotype [16]. Since inflammation

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
is considered a TH1 mediated immune response due to the abundance of IFNγ, IL-2 and NFκB activation, it is hypothesised that the inflammatory susceptible Lewis strain would also be TH1 biased. It is possible then, that the Fischer strain, being inflammation resistant, could be TH2 biased, evident in the abundance of type 2 cytokines including IL-4, IL-5, IL-10, and IL-13, with the increased activity of IκB and T-regulatory T cells (T_{REG}).

2.1.2 Glucocorticoid regulation of the immune response
Glucocorticoid regulation of cytokines is a well documented phenomenon, as is the regulation of the HPA axis by cytokines [4-6, 8, 9]. Glucocorticoids also downregulate cell proliferation through suppression of cyclin-D1 and phosphorylation of retinoblastoma protein via cyclin dependant kinase inhibitor – p21 (p21-CIP1). Glucocorticoids diffuse freely through the cell membrane and induce anti-inflammatory responses via binding to the intracellular GR; a zinc-finger transcription factor. On binding the glucocorticoid molecule the activated GR monomer forms a dimer, and translocates into the nucleus. The GR dimer promotes the transcription of a number of target genes which contain GRE motifs in their promoters [23]. Some of the genes that respond to glucocorticoid signals via GR binding to GRE include IκB, inflammation specific acute phase serum amyloid A (A-SAA) genes, SAA1 and SAA2 [24]. The IκB protein is responsible for the inhibition of NFκB, an important transcription factor involved in the transcription of TH1 cytokines [15]. By promoting the transcription of IκB and therefore blocking NFκB mediated transcription of TH1 cytokines, glucocorticoids can have an anti-TH1 effect, allowing the TH1/TH2 balance to move towards a TH2 immune response [3, 4, 14]. Interestingly, there is a negative GRE located in the promoter region of the CRH gene which is involved in the negative feedback regulation of HPA axis activity [25].

2.1.3 Inflammation and the HPA axis
The main biological role of the HPA axis is to monitor changes in stress levels and react accordingly to restore homeostasis. A major stress which activates

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?
N.O. FENNER (2012)
the HPA axis is inflammation. Inflammation is an important component of the immune response however unrestricted inflammation can be harmful. Pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα are potent activators of the HPA axis and signal immunological stress [26]. Besides cytokines, other inflammatory mediators such as catecholamines, norepinephrine, and dopamine are also involved in stimulation of the HPA axis [27, 28]. Cytokines and the HPA axis communicate bidirectionally, where cytokines influence HPA axis function and the HPA axis influences cytokine production [26]. Brain localised IL-1β has been shown to increase the activity of the HPA axis. Intracerebroventricular injections of IL-1β were shown to increase ACTH and corticosterone production. However, at higher doses Intracerebroventricular IL-1β injections resulted in downregulation of macrophage IL-1 production [26, 29]. This suggests that IL-1β is involved in HPA axis regulation and presents an inflammation-HPA axis negative feedback mechanism.

2.1.4 Immune Responses to Ovalbumin

Chicken egg white proteins, ovalbumin, have been used as a model antigen in a wide variety of immunological experiments [30, 31]. The response to ovalbumin can differ according to the route of administration and the adjuvant used [32].
Figure 2.1: Ovalbumin initiates IL-1β synthesis. Peripheral macrophages are stimulated by the injection of ovalbumin. The stimulated macrophages synthesis IL-1β, which has affects both locally and systemically. Systemic IL-1β is carried via the serculatory system and where on reception by the hypothalamus stimulated HPA axis activity.

An immune response to a given antigen can be shaped by the type of adjuvant used. Freund’s adjuvant, composed of an oil emulsion including inactivated myobacteria known to stimulate a cell mediated immune response against co-administered antigens. This results in CD8+ cytotoxic T lymphocyte (CTL) priming by phagocytes, and subsequent TNFα and IFNγ synthesis by the activated CTLs [30]. Sano et al. [31] utilised Freund’s-Ovalbumin (FO) to demonstrate the anti-allergic effects of the TH1 immune response, where eosinophil counts were reduced through FO immunisation compared to alum-ovalbumin (AO) immunisation. Therefore induction of TH1 related inflammation...
can be induced through FO immunisation whilst changing the adjuvant to alum results in TH2 related allergenic responses. A suspension preparation containing fine Aluminium hydroxide crystals (alum) can be used as an adjuvant for activating the TH2 immune response to antigens [35, 36]. Like Freunds, alum, when used as an adjuvant, can create an immune response which shapes how the accompanying antigen is processed. Alum activated TH2 immune responses are characterised by IL-4 production and IgG1 but not IgG2a synthesis. However alum activation is not dependant on IL-4 or IL-13 [35]. The mode of action of alum is poorly understood however recent research suggests that alum may act upon antigen-presenting dendritic cells directly influencing TH1 or TH2 induction [36]. Dendritic cells play an important role in Th cell differentiation (Figure 2.2), where antigen presentation is crucial. Dendritic cells may be more crucial in TH2 differentiation due to the effects of IFNγ on dendritic cell development [37]. Dendritic cells function to firstly prime naïve CD4+ cells independently of cytokines, and secondarily to assist T helper cell maturation via releasing cytokines; either IL-12, IL-18, and IL-23 for TH1 differentiation or IL-4 for TH2 differentiation [38].
T cell differentiation is determined by three factors including the extracellular cytokine environment, antigen presentation and antigen/parasite density. These factors combine to activate STAT1/STAT4 signalling resulting in autocrine IFNγ stimulation and TH1 differentiation, or STAT6 signalling resulting in autocrine IL-4 stimulation and TH2 differentiation.

Figure 2.2: How antigen presenting cells (APC), including dendritic cells, can determine T helper cell differentiation. T cell differentiation is determined by three factors including the extracellular cytokine environment, antigen presentation and antigen/parasite density. These factors combine to activate STAT1/STAT4 signalling resulting in autocrine IFNγ stimulation and TH1 differentiation, or STAT6 signalling resulting in autocrine IL-4 stimulation and TH2 differentiation.
Figure 2.3: Notch ligands instruct the fate of naïve T cell differentiation. In an extracellular environment with TH1 inducing stimuli, antigen presenting cells express delta ligands on their surface promoting TH1 cell differentiation. With an extracellular environment consisting of TH2 inducing stimuli APCs express jagged on their surface and promote the differentiation of naïve T cells into TH2 cells.

The notch pathway is able to direct T cell differentiation through ligand reception and represents another mechanism of T cell differentiation (Figure 2.3). Depending on the extracellular environment either delta or jagged is expressed on the APC surface. Reception of the delta or jagged ligand by the notch receptor directs TH1 or TH2 cell differentiation respectively. Antigen presenting cells, including dendritic cells, are also able to stimulate the differentiation direct TH2 differentiation independently of the IL-4/STAT6 pathway through the notch/jagged pathway. Jagged expression on APCs is stimulated by extracellular TH2-inducing stimuli. Expression of IL-4 and GATA3 is controlled by notch reception of jagged by naïve T cells [39].
2.1.5 Hypothesis
The HPA axis regulates glucocorticoid synthesis; therefore differing levels of HPA axis activity may influence TH1/TH2 differentiation. It is hypothesised that the immune response to specific model antigens will differ between the rat strains due to their inherent HPA axis profile.
2.2 Materials & Methods

For a more detailed explanation of methodology refer to chapter 6: materials and methods.

2.2.1 Animals

Male Fischer and Lewis rats were obtained from the Animal Research Facility (ARC) Perth. The rats were delivered at 10 weeks of age and were acclimatised for 2 weeks in the animal house at CSIRO Armidale. The animals were housed 3 per cage, and each box consisted of animals within the same strain and treatment group. Water and food were provided \textit{ad libitum}. Rats were kept in a darkened room at a constant temperature of 21°C (+/- 3°C), with filtered light periods of approximately 12 hours per day. Animals were housed and maintained according to the Animal Research Review Panel (ARRP) guidelines, with Animal Ethics Committee approval (approval number 08/07).

2.2.2 Experimental Design

\textit{Rat strains}

In order to assess the role of the neuroendocrine system in regulating the immune response to model antigens, two rat strains were selected, being the Fischer and Lewis albino brown rat (\textit{Rattus norvegicus}) strains.

\textit{Immune Challenge}

As illustrated in table 2.1, treatment groups consisted of 3 rats per group, immunised with Freund’s complete adjuvant + Ovalbumin (FO), Alum + Ovalbumin (AO) or saline control (C). Immunisations were via sub-cutaneous injection into the region between the shoulder blades (200µL total volume).
Table 2.1: Treatment Groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Freund’s + Ovalbumin</th>
<th>Alum + Ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lewis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-C</td>
<td>200µL Saline</td>
<td>L-FO 200µL Freunds + 500µg Ovalbumin</td>
<td>L-AO 200µL Alum + 500µg Ovalbumin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fischer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-C</td>
<td>200µL Saline</td>
<td>F-FO 200µL Freunds + 500µg Ovalbumin</td>
<td>F-AO 200µL Alum + 500µg Ovalbumin</td>
</tr>
</tbody>
</table>

Rats received a secondary antigen administration 35 days following the primary model antigen stimulation OVA antigen emulsified in Freund’s incomplete (FO) or OVA antigen with alum (AO). A tertiary antigen administration was given 5 days following the secondary antigen administration comprising of OVA antigen emulsified in Freund’s incomplete (FO) or OVA antigen with alum (AO). The rats were euthanased via CO$_2$ asphyxiation 4 days following the tertiary stimulation, as illustrated in Figure 2.4. As illustrated in figure 2.4 blood samples were taken over a 38 day period, where immunisations occurred on days 0, 27 and 33. Euthanasia of the animals was at day 38. Blood was sampled at days 0, 1, 2, 4, 6, 24, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, and 37.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
2.2.3 Euthanasia, animal dissection and tissue preservation

Five days following the tertiary administration of treatments, rats were euthanased via CO₂ asphyxiation. Immediately following euthanasia and cardiac puncture to attain a terminal blood sample, rats were dissected. Firstly, the head was removed and dissected. The brain was removed from the skull and the hypothalamus, hippocampus and pituitary glands dissected. These tissues were rinsed in PBS at 37°C before being snap frozen in liquid nitrogen. The pre-scapular lymph nodes and adrenal glands were removed from the animal, trimmed of excess fat and snap frozen in liquid nitrogen. The liver was removed whole and weighed to obtain the liver wet weight. For liver fat analysis, the liver was removed from the animal, divided into two samples and stored at -20°C. All tissues removed were washed in warm PBS.
2.2.4 Quantitative PCR

Quantitative PCR (qPCR) was used to measure gene expression in tissues including the lymph nodes, adrenal glands and the hypothalamus in order to measure HPA axis involvement in the immune response to model antigen challenge. RNA was isolated using Qiagen RNeasy Mini kits, utilising the Qiagen on-column DNase digestion. A further DNase digestion was performed off column using the Ambion turbo DNase kit. cDNA synthesis was performed using Invitrogen Superscript III first-strand synthesis kit. PrimerDesign housekeeping gene assays were performed to determine the optimal reference genes for use in the qPCR reactions for each tissue type and two reference genes were chosen for each tissue analysed. qPCR reactions were performed using BioRad SybrGreen supermix with the BioRad IQ5 thermal cycler real-time PCR machine.

Table 2.2: Table of Genes Used in qPCR: The expression of the genes listed for each tissue was assessed using qPCR

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Target Genes</th>
<th>Reference Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal Gland</td>
<td>GR, MC2R, P450, SF-1, StAR</td>
<td>ATP5B, GAPDH</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>GR, AVP, CRH, IL-1β, IL-1R1</td>
<td>ACTB, CANX</td>
</tr>
<tr>
<td>Pre-Scapular Lymph Node</td>
<td>GR, IFNγ, IL-2, IL-5, IL-6, IL-10, CD4</td>
<td>B2M, YWHAZ</td>
</tr>
</tbody>
</table>
2.2.5 RadiolImmunoAssays
Radioimmunoassays (RIA) were performed to measure the concentration of corticosterone in the plasma. Rats were bled at given time points (Figure 2.3) using tail vein puncture with 27G needles, collecting 200μL of blood in 1mL syringes and then transferred into eppendorf tubes containing 1μL of K$_2$EDTA to give a final concentration of 1.8mg/mL per 1mL of blood. The blood was immediately mixed with EDTA. Blood samples were transferred to 1.5mL micro-centrifuge tubes and centrifuged at 10,000g for 5 minutes at room temperature. Plasma was collected using a micro-pipette and stored at -20°C. RIAs were carried out using the MP Biomedicals ImmuChem Double Antibody Corticosterone $^{125}$ I RIA kit specific for rats and mice. The assay radioactivity was measured on the Perkin Elmer 2470 wizard$^2$ gamma counter using the MultiCalc software to generate the standard curve and infer the concentrations of the unknowns.

2.2.6 Total Extractable Lipids
Lipids were extracted from frozen liver tissue using an method adapted from Folch et al. (1957). Frozen rat livers were thawed at room temperature and weighed to three decimal places. Livers were then homogenised in a 20 fold volume of 2:1 chloroform-methanol. Samples were then filtered and the filtrate mixed with 0.74% KCl . The mixture was allowed to reseparate before removing the aqueous upper layer. Extracted lipids in solvent were air dried in a fume hood and the remaining lipids weighed to three decimal places.

2.2.7 Statistical Analysis
The raw Ct values for each target gene were normalised to two reference genes using the Biorad GenEx freeware program. The resulting expression values were then imported to GraphPad Prism, to undertake statistical analysis and generate graphs. Two-tailed Student’s t-tests were used to determine whether gene expression values differed significantly between experimental groups. Similarly, for the liver fat content groups were compared using Student’s t-test as above. Corticosterone levels were compared using repeated measures in
Graphpad prism. For all tests significance levels were set at \( \alpha=0.05 \).
Significance is represented in figures with stars * = \( p<0.05 \), ** = \( p<0.001 \) and *** = \( p<0.001 \)
2.3 Results

2.3.1 Corticosterone Response to Model Antigen Immune Stimulation

Corticosterone, the predominant glucocorticoid in rodents (cortisol is predominant in humans and sheep), is a key marker of HPA axis activity and an increase in plasma corticosterone is indicative of an increase in stress levels. Corticosterone levels measured from the control treatment groups showed that Fischer and Lewis strain rats differed in basal corticosterone levels, where Fischer had an increased level of corticosterone compared to Lewis (Figure 2.5 and Table 2.3).

Figure 2.5: Basal plasma corticosterone concentrations from the strain controls. Plasma corticosterone was measured using RIA. Statistical significant differences in basal plasma corticosterone levels are represented by * (stars). Red arrows signify where antigen challenges were given.
Table 2.3: Statistical summary of the differences in basal corticosterone levels. Plasma corticosterone was measured by RIA. The mean corticosterone values for each strain at each timepoint were compared using T-test in GraphPad prism. The resulting means and p-values are displayed in the table below.

<table>
<thead>
<tr>
<th>Vaccination Timeline</th>
<th>Difference (ng/mL)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>547</td>
<td>0.0043</td>
</tr>
<tr>
<td>29</td>
<td>311.5</td>
<td>0.0330</td>
</tr>
<tr>
<td>32</td>
<td>218.8</td>
<td>0.0455</td>
</tr>
<tr>
<td>33</td>
<td>360.7</td>
<td>0.0234</td>
</tr>
<tr>
<td>34</td>
<td>499.5</td>
<td>0.0167</td>
</tr>
<tr>
<td>36</td>
<td>378.1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Figure 2.6: Plasma corticosterone concentrations during Freunds + Ovalbumin antigen immune stimulation. Plasma corticosterone was measured using RIA. Red arrows signify where antigen challenges were given. There is no points that are significantly different from control.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Although basal corticosterone was shown to differ between strains, there was no significant change in corticosterone levels within strains in response to FO model antigen immune vaccination (Figure 2.6).

Plasma corticosterone levels changed in both Fischer and Lewis strain rats in response to AO model antigen immune stimulation (Figure 2.7).

The Fischer strain response to AO significantly differed at certain time points during the immune stimulation regime. Corticosterone was reduced after day 1 following the first vaccination (vac. timeline day 0), where the treatment group was 294.5ng/mL less (p=0.0184) than the control. However by day 6 (vac. timeline day 6) corticosterone had increased to be 183.3ng/mL more (p=0.0448) than the control. There were no significant differences during the second vaccination. During the third vaccination corticosterone levels were significantly lower in the treatment group than the control. Similar to the primary vaccination, corticosterone levels were 428.8ng/mL lower (p=0.0421) at day one of the third vaccination (vac. timeline day 34) and 359.6ng/mL lower (p=0.0433) at day 3 (vac. timeline day 36).

The reduction in plasma corticosterone following AO immune stimulation was less marked in rats from the Lewis strain. At 24 days following the primary vaccination (vac. timeline day 24) the Lewis AO treated rat’s mean corticosterone was 219.6ng/mL (p=0.0145) less than the mean for the Lewis control group.
Figure 2.7: Plasma corticosterone concentrations during Alum + Ovalbumin antigen immune stimulation. Plasma corticosterone was measured using RIA. Differences in plasma corticosterone levels in response to antigen challenge group from control group are represented by * (stars). Red arrows signify where antigen challenges were given.

2.3.2 Gene Expression Changes in the Prescapular Lymph Node in Response to Model Antigen Immune Stimulation

Gene Expression of Type 1 Cytokines in the Prescapular Lymph Node in Response to Model Antigen Immune Stimulation

The prescapular lymph node (PLN) is the draining lymph node for the subcutaneous injection site used in the experiments described in this chapter. Levels of selected gene mRNAs were measured using qPCR performed upon RNA extracted from whole PLNs dissected from the rats immediately post mortem.

The inflammation susceptible Lewis rats had higher levels of INFγ mRNA in

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N. O. FENNER (2012)
PLN in response to administration of either FO or AO, (Figure 2.10) and had a high basal level of IL-2 mRNA, compared to the inflammation resistant Fischer strain (Figure 2.11).

IFNγ is a key cytokine in the TH1 immune response. Synthesised by TH1 cells, IFNγ switches monocyte differentiation from dendritic cells to macrophages through autocrine macrophage colony stimulating factor (M-CSF) and IL-6 production [37] and stimulating IL-1β production. IFNγ is also involved in B cell antibody class switching. B cells stimulated with bacterial LPS, in the presence of IFNγ, secreted IgG2a, but secreted less IgG3, IgG1, IgG2b and IgE, whilst IgM was unaffected. This showed that IFNγ acted directly on B cells to affect antibody production and class [41]. IFNγ gene expression in the prescapular lymph node of Lewis rats was upregulated in response to model antigen immune stimulation (Figure 2.9). IFNγ was upregulated by 2.3 fold (p=0.0281) and 3.5 fold (p=0.0333) in the freunds + ovalbumin (FO) and Alum + ovalbumin (AO) groups respectively, compared to the control. There was no significant change in IFNγ gene expression seen in the Fischer strain. There was no difference in basal levels of IFNγ gene expression between the Lewis and Fischer control groups (p=0.104).

IL-2 is an important type 1 cytokine synthesised by TH1 cells. IL-2 plays an important role as a growth factor for the stimulation, growth and proliferation of T and B cells, and is also important in the maintenance of T\text{REG} cells. IL-2 gene expression in the prescapular lymph node was downregulated in the Lewis strain in response to freunds adjuvant, but not alum, and differences in basal levels of expression were observed between strain controls. There were also strain differences in immune response to FO. IL-2 was significantly downregulated by 1.8 fold (p=0.0382) in the Lewis strain following FO exposure compared to the control, but not from AO exposure (figure 2.11). Basal gene expression of IL-2 was 2.2 fold (p=0.0173) higher in Lewis control compared to Fischer control. There is no significant change in IL-2 gene expression in the Fischer strain.
**Gene Expression of Type 2 Cytokines in the Prescapular Lymph Node in Response to Model Antigen Immune Stimulation**

The expression of type 2 cytokine genes in the prescapular lymph node was also measured in response to model antigen immune stimulation. The inflammation resistant Fischer rats had a 24.2 fold (p=0.0086) lower basal level of IL-5 (control group) than the Lewis rats. There was no significant IL-5 gene expression change in response to antigen administration observed in either Lewis or Fischer strain rats to either AO or FO (figure 2.12). IL-5 is synthesised by mast cells and TH2 cells and plays an important role in B cell proliferation and eosinophil activity.

As an anti-inflammatory cytokine, IL-10 is important in the TH2 immune response. As well as increasing B cell survival and proliferation IL-10 also down-regulates the TH1 immune response. A significant 1.9 (p=0.0247) fold increase in IL-10 gene expression was observed in Fischer rats in response to AO, but not FO (Figure 2.14). In contrast, there was no increase in IL-10 gene expression in Lewis rats in response to either AO or FO, nor were there basal differences in expression between Fischer and Lewis controls.

**Gene Expression of the Glucocorticoid receptor in the Prescapular Lymph Node in Response to Model Antigen Immune Stimulation**

The GR is an important transcription factor for anti-inflammatory gene expression. GR binds glucocorticoids and initiates the action of glucocorticoids at cellular level when the GR-glucocorticoid molecules dimerise, enter the cell nucleus and promote gene expression via binding to GRE motifs. Analysis of the gene expression of GR showed that the Lewis rat strain had a significantly reduced level of GR gene expression compared to the Fischer, with the GR mRNA transcript of Fischer being 1.9 fold more abundant than that of the Lewis (p=0.028, Figure 2.9).
Figure 2.8: CD4 gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.9: GR gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.10: IFNγ gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. IFNγ gene expression was upregulated in the Lewis strain in response to both antigen stimulations. There was no change in expression in the Fischer group. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.11: IL-2 gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. IL-2 expression was downregulation in the Lewis FO treatment group. Lewis basal expression of IL-2 was higher than Fischer. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.12: IL-5 gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. Basal IL-5 gene expression was higher in the Lewis control group than the Fischer control group. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * $p<0.05$, **$p<0.01$, ***$p<0.001$. SEM represented by error bars.
Figure 2.13: IL-6 gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.14: IL-10 gene expression in the prescapular lymph nodes in response to model antigen immune stimulation. IL-10 expression was upregulated in the Fischer AO group compared to Fischer control. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
2.3.3 Gene Expression in the Hypothalamus in Response to Model Antigen Immune Stimulation

The expression of genes in the hypothalamus were measured using qPCR. The genes chosen were some of the genes central to HPA axis function in these tissues. It was shown that the inflammation resistant Fischer strain expressed a higher level of CRH in the hypothalamus (p=0.044, Figure 2.16), but did not increase expression of any of the genes studied in response to FO. The inflammation susceptible Lewis strain did not change gene expression significantly in response to AO or FO.

There was no significant difference in gene expression of the genes assayed in the hypothalamus between the controls of the Fischer and Lewis strains.

CRH is a small polypeptide secreted from the paraventricular nucleus of the hypothalamus and is a central component of HPA axis signalling. Binding to CRH-R at the anterior pituitary initiates synthesis of ACTH from POMC cleavage. Of rats stimulated with AO, CRH in the Fischer but not Lewis strain was upregulated by 1.8 fold in comparison to the control (p=0.044) CRH expression did not change in response to either model antigens in the Lewis strain. There was no difference in basal expression of CRH between the Fischer and Lewis controls.
Figure 2.15: AVP gene expression in the hypothalamus in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.16: CRH gene expression in the hypothalamus in response to model antigen immune stimulation. CRH expression was upregulated in the Fischer AO group compared to the Fischer control group. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.17: GR gene expression in the hypothalamus in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.18: IL-1β gene expression in the hypothalamus in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.19: IL-1R1 gene expression in the hypothalamus in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
4.3.4 Gene Expression in the Adrenal Gland in Response to Model Antigen Immune Stimulation

The expression of genes in the adrenal glands were measured using qPCR. The genes chosen were some of the genes central to HPA axis function in these tissues. There was no significant difference in gene expression of the genes assayed in the adrenal gland between the controls of the Fischer and Lewis strains.

ACTH is synthesised, cleaved and secreted into the peripheral blood supply by corticotroph cells. ACTH binds to MC2R on the surface of cells of the adrenal cortex, initiating corticosterone synthesis. The expression of MC2R was upregulated by 2.1 fold (p=0.009) in response to AO model antigen immune stimulation but not FO in the Fischer strain (figure 2.21). There was no change in MC2R gene expression observed in the Lewis strain response to model antigens. There was no significant difference in the basal gene expression of MC2R between the Fischer and Lewis controls.
Figure 2.20: GR gene expression in the adrenal gland in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.21: MC2R gene expression in the adrenal gland in response to model antigen immune stimulation. MC2R expression was upregulated in the Fischer AO group compared to Fischer control Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.22: P450 gene expression in the adrenal gland in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.23: SF-1 gene expression in the adrenal gland in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
Figure 2.24: StAR gene expression in the adrenal gland in response to model antigen immune stimulation. There were no significant gene expression changes in response to antigen stimulation. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * p=<0.05, **p=<0.01, ***p=<0.001. SEM represented by error bars.
2.3.5 Other physiological observations

Liver Lipid Content

Fat metabolism is affected by glucocorticoids and this is evident in Cushing’s syndrome and prolonged corticosteroid usage where an increase in glucocorticoids correlates with increased fat storage in livers. This phenomenon was observed in the experiment by measuring the total extractable lipids from the livers extracted from each rat in the experimental groups (Figure 2.25). The Fischer control group had 6.2% more (p=0.01) liver fat deposited than the Lewis control. Similarly, there were significant differences between strains within the treatment groups with Fischer FO having 5.0% more (p=0.003) fat deposited than Lewis FO, and Fischer AO having 6.3% more (p=0.01) fat deposited than Lewis AO. No significant difference in percentage liver fat was observed between experimental groups within each rat strain, showing that the experimental treatments had no effect on fat deposition in the liver.
Figure 2.25: The percentage total extractable liver lipids. The percentage lipids of total liver wet weight was measured by chloroform extraction as described by Folch et al. (1956). The Fischer control had a higher level of liver lipids than Lewis group. Fischer treatment groups are represented by the yellow colour group, while the blue group represents the Lewis treatment groups. Significant differences are signified by * (stars) with lines linking the relevant treatment groups. * $p<0.05$, **$p<0.01$, ***$p<0.001$. SEM represented by error bars.

Body Temperature

Changes in body temperature can be indicative of inflammation and are a trigger for HPA axis activity. By measuring body temperature of the rat treatment groups over the course of the vaccination regime, differences in body temperatures were expected. Rat temperatures were measured using digital rectal thermometers (Figure 2.26 & 2.27). There were no significant differences in rectal temperatures in response to model antigen vaccination with respect to the controls.
Figure 2.26: Temperature regulation in response to model antigen immune stimulation. Red arrows represent days were vaccinations are given. There are no significant differences between treatment and control groups in response to antigen stimulation.
2.4 Discussion

The interaction between inflammation, glucocorticoid activity and T helper cell differentiation presents a regulated mechanism for a tailored immune response and may be one of many specific mechanisms responsible for dictating T helper cell differentiation. Other factors influencing T helper cell differentiation include APC type, antigen density and pattern recognition receptors.

The complement pathway is a classical innate pathway, where complement factor B synthesis is upregulated in response to LPS or dsRNA, which subsequently activates the transcription factor NFκB [43]. Glucocorticoids are known to inhibit the activity of NFκB through IκB mediated suppression. This suggests that the complement pathway may be tailored towards the TH1 response where inflammation is crucial in the early stages of the response.

In these experiments evidence supporting HPA axis mediated immune regulation was limited; however there was some evidence which suggested that the HPA axis may influence type 1 and 2 cytokine expression. This was seen in the up-regulation of IFNγ and IL-2 in L-AO, whilst IL-10 was up-regulated in F-AO. Basal levels of cytokine expression were also shown to differ due to HPA axis activity with IL-2 and IL-5 expression being higher in the L-C than the F-C.

Observations that Fischer is inflammation resistant whilst Lewis is inflammation susceptible are common in the literature [16, 22]. These observations were supported in this study with Fischer showing a tendency towards anti-inflammation characterised by expression of anti-inflammatory type 2 cytokines. This was in response to presentation of the immune system with an antigen/adjuvant formulated to stimulate a type 2 response. Lewis, when presented with an antigen/adjuvant aimed at stimulating a type 1 immune response, showed a tendency towards inflammation. This was characterised by increased expression of the type 1 cytokine IFNγ. Interestingly, when Lewis
were presented with a type 2 antigen/adjuvant the response was similar to the type 1 response in terms of IFNγ expression, supporting that Lewis are susceptible to inflammation.

Corticosterone responses over the course of the trial were not as expected, with the Fischer strain down-regulating plasma corticosterone levels in response to AO (type 2 antigen/adjuvant). Whilst the gene expression suggested an up-regulation in HPA activity evident through increased expression of CRH and MC2R, however Fischer plasma corticosterone levels were decreased to a level not significantly different from Lewis basal levels. It is hypothesised that this result may be due to a number of factors including an additional level of regulation at the gene translational stage as an effect of corticosterone negative feedback into the HPA axis. Similarly, the upregulation in gene transcription may be a mechanism in equipping the system to respond in a greater magnitude in the event of further inflammatory insults. To test these theories both gene expression and protein levels need to be studied in detail, as well as a corresponding experiment examining differing levels of antigen with a varying time course.
Figure 2.28: Hypothesised mechanism behind inflammation susceptibility and inflammation resistance in Lewis and Fischer rat strains respectively. The relative abundance of extracellular glucocorticoids has an effect on how T cells react in terms cytokine type produced. In the absence of glucocorticoids, NF-κB is uninhibited by IκB, allowing for the transcription of type 1 cytokines, whilst in the abundance of glucocorticoids IκB inhibits the activity of NF-κB. IκB synthesis is initiated through GR transcriptional activity (Fenner 2011)

The main mechanism behind inflammation susceptibility of the Lewis strain is hypothesised to involve the interactions of NFκB with IκB (outlined in figure 2.28). The level of glucocorticoids and the activity of NFκB exist in a state of equilibrium. Low levels of glucocorticoids allow for the ubiquination of the NFκB repressor protein, IκB. This liberates NFκB which promotes the expression of type 1 cytokines (pro-inflammatory cytokines). The reverse is in times of high glucocorticoid levels where the activated GR promotes IκB expression which is able to actively repress the activity of NFκB, therefore repressing pro-
inflammatory cytokine expression and subsequently allowing type 2 cytokine (anti-inflammatory cytokines) expression to occur unhindered. The low abundance of glucorticoids as seen in the Lewis strain shifts the equilibrium towards the expression of pro-inflammatory cytokine, resulting in inflammation susceptibility. It is also hypothesised that during a parasitic immune response the equilibrium may shift from at first being pro-inflammatory as an innate immune response to being anti-inflammatory as the body begins glucocorticoid synthesis and a more specific and antibody producing adaptive immune response.

Besides the use of adjuvants to influence how the immune system responds, the concentration of antigen can also dictate T helper cell differentiation. Low and high concentration of antigen may result in TH1 responses whilst moderate concentration levels may result in TH2 response. Also, fully differentiated T cell may also response to changes in antigen concentration, where TH1 cells respond to moderate antigen levels and TH2 cells respond to a wide range of concentrations. The mechanism behind this is antigen presentation [44].

Although no specific antigen presenting cells where targeted in this study, the role of basophils in T helper cell differentiation may be of interest in future experiments. Basophils are able to act as antigen presenting cells in certain conditions where presentation is coupled with IL-4 production driving TH2 differentiation [45-47].

If this experiment was to be repeated it is recommended that the antigen or adjuvant used is strong enough to initiate a HPA axis response in terms of increased corticosterone synthesis and also have antibodies titres measured using ELISA or similar, to further differentiate the responses to the two antigens. Also it may be interesting to study the effects of the administration of synthetic corticosterone, in the form of dexamethasone (DEX) for example, during the vaccination regime to determine whether gene expression and T helper cell differentiation may be affected. To further study the hypothesis that basal levels of corticosterone in rats are important in shaping the immune response, rats of
the same strain and having similar corticosterone levels may be used analyse this further. Rats would be subjected to a DEX regime artificially establishing differing levels of plasma corticosterone. The above vaccination regime could then be applied and the effects measured. This would demonstrate a role for basal levels of corticosterone in immune responses to simple antigens.

Despite not seeing a comprehensive neuroendocrine response to model antigen vaccination, this experiment did suggest that the basal level of HPA axis activity is important in shaping the immune response. The model antigens used remain in the animal's system for a much shorter period than a live infection such as a parasite, where chronic infections are common. This may have been a factor with the short time frame not allowing enough time to result in a strong HPA axis response. These factors combined could have contributed to the lack of HPA axis response, seen in the lack of increased production of corticosterone. Also, the brief exposure to antigen achieved with a subcutaneous injection may not have been sufficient to cause immunological stress.

From this experiment it was evident to an extent that the basal level of HPA axis activity was a contributing factor in influencing the cytokine profile. This was demonstrated using simple antigens and manipulated using adjuvants. To further the understanding of the role the HPA axis has in shaping the immune response, an experiment where a parasite is used to cause immunological stress will be utilised. As parasites commonly cause polarisation of the T helper response, towards the TH2 response characterised by type 2 cytokine expression, this following experiment will determine whether the cytokine profile changes seen in this chapter are reproducible given the change in immune stressor. This chapter showed that Fischer and Lewis strains differ in their response to model antigen immunological stressors, where the Lewis strain demonstrated a greater susceptibility to inflammation, whilst the Fischer strain showed a tendency towards TH2 mediated immune responses. From these
observations it is hypothesised that the Lewis strain may demonstrate a greater susceptibility to parasite infection, whilst the Fischer strain may be more resistant due to the HPA axis mediated regulation of the immune response.

2.5 References

15. . !!! INVALID CITATION !!!
19. Dhabhar FS, Miller AH, McEwen BS, Spencer RL. Differential activation of adrenal steroid receptors in neural and immune tissues of Sprague Dawley, Fischer 344, and Lewis rats. Journal of...
30. Snapper CM, Peschel C, Paul WE. IFN-gamma stimulates IgG2a secretion by murine b cells

**Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?**

**N.O. FENNER (2012)**
CHAPTER 3: Differences in the Primary Immune Response and Subsequent HPA Axis Response to Heligmosomoides bakerii between Lewis and Fischer Rat Strains

3.1 Introduction

The T cell mediated immune response is classically divided into two subtypes. The TH1 immune response is characterised by type 1 cytokine synthesis (e.g. IFNγ), persistent inflammation and is evident during the cellular immune response effective at removing bacterial and viral antigens. On the other hand, the TH2 immune response is characterised by type 2 cytokine production (e.g. IL-4), acute and sometimes systemic inflammation including the activation of mast cells and development of IgE producing B-cells, is seen in allergic reactions and is involved in the immune response to parasites. In this chapter we study the effects of high and low corticosterone profiles on the immune response to Heligmosomoides bakerii utilising the inflammation resistant, high basal corticosterone rat strain Fischer, and the inflammation susceptible, low basal corticosterone rat strain Lewis. This study aims to determine whether basal corticosterone levels have an effect on the immune response to parasites, by studying gene expression from multiple tissues involved in the immune-neuroendocrine interface, confirming gene expression, measuring corticosterone levels during parasite challenge, determining differences in cell populations between strains during challenge, and measuring physiological aspects of parasite challenge.

These experiments were conducted to determine if differences in corticosterone levels exhibited by the two rat strains would affect the type of immune response mounted. Fischer rats have higher corticosterone than Lewis and so inflammation in response to infection was predicted to be lower in Fischer than Lewis. This was expected to be shown through increased levels of pro-
inflammatory and type 1 cytokines in the Lewis, relative to the Fischer rats. Corticosterone synthesis is controlled through the negative feedback influenced HPA axis and it is here that we expect to observe differences between the strains demonstrating that differences in the neuroendocrine responses are relative to corticosterone synthesis. The relationship between cytokines, glucocorticoids and the function of the neuroendocrine system is well documented [1-3]. Changes to and breakdowns in HPA axis signalling can have profound effects on the effective responsiveness of the HPA axis to stressors, resulting in altered glucocorticoid synthesis. This is evident in experiments involving adrenalectomy [4]. Changes in HPA axis responsiveness to stressor would ultimately affect glucocorticoid synthesis which in term would have an effect on immune function. Glucocorticoids are known to be anti-inflammatory, directly affecting the activity of NF-κB through promotion of the repressive IκB molecule. This results in repression of inflammation and the suppression of the TH1 immune response. However, the TH2 immune response, essential in anti-parasite immune responses, seems to be unaffected by glucocorticoids. Therefore, it was expected that Fischer would mount and sustain a more appropriate immune response to *H. bakerii* due to the availability of glucocorticoids and more specifically IκB, therefore demonstrating that the neuroendocrine system could be involved in regulating the immune response to parasitic nematodes.

*H. polygyrus* is a natural parasite of mice. However, recent research suggests that the laboratory isolated strain of *H. polygyrus* has diverged under laboratory selection and an alternative species name is suggested for the laboratory-bred *Heligmosomoides: H. bakerii* [5].

### 3.1.1 Rodent immune responses to *H. bakerii*

*H. bakerii* is a natural parasite of mice. Infection of mice involves the injection of larvae which once ingested migrate through the gastrointestinal tract residing in the duodenum and the proximal jejunum [6, 7]. At this stage the larvae burrow into the lumen where moultling occurs. The worms emerge from the lumen to...
sexually reproduce shedding viable eggs into the gastrointestinal flow. The eggs are released to the environment in faecal matter where infective larvae hatch and await consumption. Various strains of mice show differences in responsiveness to *H. bakerii* parasitism. High responder strains include SJL, SWR, mid-range responders include BALB/c, NIH, B10G, and DBA, and low responders include CBA & C3H C57bl/10 [8]. It is thought that the expulsion of parasites from the low responders is due to the completion of the worm’s life span and not immune functionality [8]. Resistance exhibited by the SJL strain is a combination of the speed and magnitude of the immune response, where CD4\(^+\) cells play a crucial role in activating neutrophils [8] and regulating IgE production [9]. An effective immune response to *H. bakerii* is dependant on the TH2 immune response. Whilst T lymphocytes play a crucial role in establishing and regulating the TH2 immune response, B lymphocytes are also a crucial component of this response. B lymphocytes are known to produce TNF\(\alpha\), stimulating the TH1 response and IL-10, regulating the TH2, with emerging evidence suggesting that B cells are able to determine the magnitude of the CD4\(^+\) immune responses [10]. By studying the role of B cells in the context of parasitological challenge, B cells were shown to be a crucial component of the effective immune response to *H. bakerii*, through antibody production and enhancing expansion and differentiation of both primary and memory TH2 cells [10]. Results obtained also demonstrated that B cells are also capable of contributing to the immune response by producing regulatory cytokines TNF\(\alpha\) and IL-2 which regulate the quality and magnitude of the immune response [10]. In fact, the difference between fast and slow responders may, in part, be determined by the both the number and duration of B cells at the mesenteric lymph node [11]. IgG1 antibody is important in the primary response to *H. bakerii* where high antibody serum concentrations are typical of acute and intermediate infections, but not chronic infections. Also antibody serum concentration was not affected by worm burden. This demonstrates that B cells are responsible for the production of IgG1 with the serum concentration determined by infection time not intensity [12].

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

99
Figure 3.1: Lifecycle of *H. ploygyrus* in mice. Mice are the natural host for the parasite *H. polygyrus*. Mice become infected with larvae contaminating their food. On entry into the host via ingestion, larvae exsheath and pass passively down the gastrointestinal tract to the duodenum and the proximal jejunum. Here the larvae burrow into the intestinal mucosa where they begin to mature. On maturation they emerge into the lumen and breed. Eggs are passed in the faeces where they hatch and the larvae disperse into the environment (figure copyright Fenner 2011).

3.1.2 Rat Immune Response to *H. bakerii*

*H. bakerii* has also been shown to cause an inflammatory response in rats but is unable to reproduce [13-17]. Whilst able to penetrate the rat gastrointestinal mucosal tissue, an intense inflammatory response is generated causing extensive connective tissue generation and rendering the larvae unable to re-emerge as seen in the mouse [13, 14]. The inflammatory response seen in the rat can be ablated through corticosterone treatment, which results in a response similar to that of the mouse where the larvae re-emerge and are able to reproduce [13]. Therefore, glucocorticoids are important in the susceptibility of rats to *H. bakerii* infection. Inflammation in this instance is crucial for resistance,
at least initially, causing entrapment of the larvae in the gut mucosa through connective tissue genesis.

Figure 3.2: *H. polygyrus* infection in rats. Rats are not the natural host of *H. polygyrus* however do cause pathological damage to the gastrointestinal tract. They are unable to produce eggs except in immuno-suppressive circumstances. The infection timeline involves the ingestion of larvae where the larvae migrate to the gastrointestinal tract where they burrow into the mucosal tissue as seen in the mouse. However, an effective immune response is then mounted and the larvae are trapped through connective tissue genesis. The larvae are unable to emerge into the gastrointestinal tract and breed and therefore are killed within the rat GI tissue. (figure copyright Fenner 2011)

**3.1.3 Rodent regulation of immune responses to gastrointestinal parasites**

Research into the immune response of mice to *H. polygyrus* and *H. bakerii* is well established. The immune response is predominately TH2 with a predominate role for antibodies, especially IgG1, in effective immune responses [12, 18, 19]. The immune response has been documented to be regulated in part by cytokine producing B cells [10]. The anti-inflammatory effects of this

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
response is well documented, where *H. polygyrus* infections were used to reduce inflammation in chemically induced colitis [20]. Of mice vaccinated with excretory/secretory, infective larvae showed retarded development as a result [21]. Similarly, immunity against *H. polygyrus* can be achieved through subcutaneous vaccination of mice with larvae that are post-infective [22]. In addition T cell proliferation can be achieved through the inoculation of mice with adult worm homogenate [23, 24].

3.1.4 Aims & Hypothesis
This chapter aims to examine immune and neuroendocrine responses of Fischer and Lewis strain rats to the gastrointestinal nematode *H. bakerii*. Considering neuroendocrine activities affect the response of the immune system, it is hypothesised that there will be differences in immune and neuroendocrine responses to *H. bakerii* as there is a difference in the neuroendocrine activities of the two strains.
3.2 Materials & Methods
For a more detailed explanation of methodology refer to chapter 6: materials and methods

3.2.1 Animal Trial, Worm Dosing and Worm Passage

*H. bakerii* L3 larvae were obtained from the Australian National University (Associate Professor Carol Behm). The larvae were passaged through 10 CBA mice obtained from Animal Research Centre, Perth and parasite passage was approved by the CSIRO F.D. McMaster Animal Ethics Committee, (approval number: 09/04). Faecal material was collected and the parasite cultured using methods supplied by ANU. Faecal material with positive faecal egg counts (FECs) were collected in by leaving mice overnight on a mesh-floored cage. The faeces were washed and separated from other matter, before being macerated and incubated in filter paper lined petri dishes at 21°C. Larvae were collected after 5 days of incubation and washed several times until water cleared. The remaining solid material was applied to a Baermann Apparatus and the larvae collected and stored at 4°C in a tissue culture flask.

The animal trial involved 12 male Fischer rats and 12 male Lewis rats. All rats were age matched and were 12 weeks of age at the time of parasite dosing. Rats from each strain were divided into two experimental treatment groups, six receiving parasite infections and six remaining uninfected. Rats were infected for a period of 6 days before euthanasia. Rats in the infected groups received 4,000 motile L3 *H. bakerii* larvae orally in a volume of 0.5mL using a blunted needle and a 1mL syringe, whilst control rats received a saline substitute. Rat blood samples were collected at various timepoints via tail vein puncture into a 1mL syringe and then transferred into a 0.5mL tube containing EDTA. Rats were restrained during the blood sampling process in a custom-made restraining tube. At day 6 of infection rats were euthanased by CO₂ asphyxiation. Tissues were removed and frozen in liquid nitrogen and stored at -80°C for RNA isolation, stored at room temperature in 10% buffered formalin for histology, gastrointestinal tissue stored in 100% ethanol at room temperature for DNA
extraction, liver samples were frozen at -20°C for lipid analysis.

3.2.2 Quantitative PCR
RNA was isolated using Qiagen RNeasy Midi Kit (cat: 75144), using Qiagen RNase-free DNase Set for use with RNeasy/QIAamp columns (cat: 79254). A further round of DNase digestion was performed using Ambion turbo DNase-free (cat: 1907). RNA was then reverse transcribed and cDNA synthesised using Invitrogen Superscript III first-strand synthesis supermix (cat: 18080-400). Aliquots of cDNA were stored at -20°C until needed. Assay optimisation and assay quantification using real-time PCR detection was performed using the BioRad iQ5 multicolour real-time PCR detection system, using BioRad iQ sybr green supermix (170-8882). Quantitative PCR assays were normalised to expression levels of two reference genes per tissue. Pairs of reference genes were used for each tissue, and the appropriate genes were chosen following the geNorm protocol.

Table 3.1: Reference genes were selected for each tissue type using the geNorm protocol.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ref1:</th>
<th>Ref2:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal Gland</td>
<td>ACTβ</td>
<td>ATP5B</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>ATP5B</td>
<td>CANX</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>ACTβ</td>
<td>CANX</td>
</tr>
<tr>
<td>Mesenteric Lymph Node</td>
<td>B2M</td>
<td>YWHAZ</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>GAPDH</td>
<td>ACTβ</td>
</tr>
<tr>
<td>Spleen</td>
<td>ATP5B</td>
<td>CYC1</td>
</tr>
</tbody>
</table>

3.2.3 Corticosterone RadioImmuno Assay
Rats were bled via tail vein puncture and ~200μL blood was placed in 0.5mL micro-centrifuge tubes containing ~3μL EDTA (0.36mg in total) to obtain a final EDTA concentration of 1.8mg/mL in blood. Blood was separated by centrifugation at 10,000G and plasma stored at -20°C in 0.5mL micro-centrifuge tubes. Plasma corticosterone assays was established using MP Biomedicals ImmuChem double antibody Corticosterone $^{125}$I RIA kit for rats and mice kt (cat: 07120103) and measured using the Elmer 2470 wizard$^2$ gamma counter using the MultiCalc software to generate the standard curve and infer the
concentrations of the unknowns.

3.2.4 Histology
Tissues were excised at time of kill and fixed in 10% formalin. Tissues were then embedded in wax and sectioned onto positively charged glass slides. Tissues were stained using the standard procedure outlined in the methods section and viewed using a light microscope.

3.2.5 Statistical Analysis
The raw Ct values for each target gene were normalised to two reference genes using the Biorad GenEx freeware program. The resulting expression values were then imported to GraphPad Prism, to undertake statistical analysis and generate graphs. Two-tailed Student’s t-tests were used to determine whether gene expression values differed significantly between experimental groups. Similarly, for the liver fat content groups were compared using Student’s t-test as above. Corticosterone levels were compared using repeated measures in Graphpad prism. For all tests significance levels were set at α=0.05. Significance is represented in figures with stars * = p<0.05, ** = p<0.001 and *** = p<0.001.
3.3 Results

3.3.1 Hippocampus Gene Expression in Response to *H. bakerii* Infection

The presence of a high number of GR and the synthesis of ACTH secretagogues in the hippocampus has lead to it’s implementation in regulatory HPA axis feedback [25, 26]. Although not playing a significant role in HPA axis activation the hippocampus is a known site for the expression of IL-1β and IL-1R with the brain [26-28]. The hippocampus, located in the medial temporal lobe of the brain of mammals, is responsible for spatial navigation and memory. To determine whether the hippocampus played a role in the regulation of the HPA axis in response to parasites CRH, GR, IL-1β and IL-1R1 gene expression was measured. It was found that the signalling molecules CRH and IL-1β were differentially expressed in response to parasite infection, however the expression of the receptors GR and IL-1R1 remained unchanged. It is evident that if IL-1β is acting as a messager molecule, signalling inflammation and immunological stress, it is an active pathway in both strains. However, the difference in the two strains, seen at the level of corticoisterone responsiveness may lie with the fact that CRH was only upregulated in the Fisher only, despite the IL-1β signalling pathway being operable in both strains. This suggests that the hippocampus may be an important tissue responsible for the differences in neuroendocrine activity observed between the two strains. Furthermore, it is evident that IL-1β signalling in the hippocampus is conserved between the two strains but the processes after this do not translate into CRH expression in the Lewis unlike in the Fischer. Also, the results suggested that the regulatory HPA axis response seen at the hippocampus level was temporary; suggesting that regulation of HPA axis function was at the level of signalling molecules, whilst receptor expression remained constant.
Figure 3.3: Hippocampus CRH gene expression in Fischer and Lewis rats in response to *H. bakerii*. CRH expression was upregulated in response to parasites in Fischer rats. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

Expression of CRH mRNA was increased by 45% (p=0.003) in response to parasite infection in the Fischer rats, but no significant change in CRH expression was seen in the Lewis strain. When averaged over the infected and uninfected groups, there was no significant difference in CRH expression between strains.
IL-1 beta

Figure 3.4: Hippocampus IL-1β gene expression in Fischer and Lewis rats in response to *H. bakerii*. IL-1β expression was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of IL-1β was upregulated significantly in response to parasite infection in both strains. IL-1β expression was upregulated by 436% (p=0.018) in response to parasite infection in the Fischer strain. Similarly, IL-1β expression was upregulated by 160% in the Lewis strain in response to parasite infection. There was no significant difference between strains.
Figure 3.5: Hippocampus GR gene expression in Fischer and Lewis rats in response to *H. bakerii*. Expression of GR was unchanged in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant difference in the expression of GR of both strains in response to parasite infection. There was no significant difference between strains.
Figure 3.6: Hippocampus IL-1R1 gene expression in Fischer and Lewis rats in response to *H. bakerii*. Expression of IL-1R1 was unchanged in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=*p<0.05, **=*p<0.01, and ***=*p<0.001).

There was no significant difference in the expression of IL-1R1 of both strains in response to parasite infection. There was no significant difference between strains.
### 3.3.2 Hypothalamus Gene Expression in Response to *H. bakerii* Infection

The hypothalamus is the major brain tissue responsible for monitoring and maintaining homeostasis. The hypothalamus is the central organ in the regulation of stress through CRH secretion and glucocorticoid negative feedback. IL-1β levels have been shown to increase in the brain in response to immunological stress [28]. The hypothalamus is regarded as a primary site of IL-1β action in the brain, received through the activity of its receptor IL-1R1. IL-1R1 expression is upregulated in response to changing IL-1β levels [29]. Negative feedback is mediated through GR as well as some other brain functions [30-32]. As with the hippocampus CRH, GR, IL-1β and IL-1R1 gene expression in the hypothalamus was measured using qPCR. As with the hippocampus, IL-1β expression was upregulated in both strains in response to parasites while strain specific changes in GR and CRH expression levels were seen in the hypothalamus. However, unlike the hippocampus, there was upregulation of IL-1R1 receptor in response to parasitism, suggesting that the neuroendocrine response to parasite infection differs within brain tissues. Similarly, regulation of the stress response may be a sort lived process for the hippocampus while a stronger, more robust response is seen in the hypothalamus evident in the change of receptor expression of GR and IL-1R1.
Figure 3.7: Hypothalamus CRH gene expression in Fischer and Lewis rats in response to *H. bakerii*. CRH expression was upregulated in Lewis rats only in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of CRH was upregulated in the hypothalamus in response to parasite infection by 35% (p=0.048) in the Lewis strain only. There was no significant difference in CRH expression in response to parasite infection in the Fischer strain.
Figure 3.8: Hypothalamus GR gene expression in Fischer and Lewis rats in response to *H. bakerii*. GR expression was upregulated in response to parasites in Fischer only. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The GR was upregulated in the hypothalamus in the Fischer strain only by 50% (p=0.03). There was no significant difference in GR expression in response to parasite infection in the Lewis strain. There was no difference in GR expression between strains.
Figure 3.9: Hypothalamus IL-1β gene expression in Fischer and Lewis rats in response to \textit{H. bakerii}. IL-1β expression was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

Expression of IL-1β in the hypothalamus was similar to that of the hippocampus, where both strains showed significant upregulation. IL-1β was upregulated in the hypothalamus by 217% (p=0.004) in the Fischer strain and 209% (p=0.00003) in the Lewis strain. However, there was no significant strain differences in IL-1β expression.
Figure 3.10: Hypothalamus IL-1R1 gene expression in Fischer and Lewis rats in response to *H. bakerii*. IL-1R1 was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

Unlike the hippocampus, IL-1R1 was upregulated in both strains in response to parasite infection. In the Fischer strain, IL-1R1 was upregulated by 56% (p=0.042), whilst in the Lewis strain IL-1R1 was upregulated by 74% (p=0.037) in response to parasite infection. There were no significant strain differences.
3.3.3 Adrenal Gland Gene Expression in Response to H. bakerii Infection

The adrenal gland is the main site of glucocorticoid synthesis. Glucocorticoids are synthesised in a series of biochemical reactions converting cholesterol into corticosterone. The main rate limiting enzyme in this reaction is the cytochrome P450\text{SCC}. The signalling process originating from the hypothalamus and pituitary terminates with the reception of ACTH at the MC2R. The reception leads to the synthesis of glucocorticoids. The translocation of steroidogenic precursors from the outer to the inner mitochondrial membrane of the adrenal cortex is catalysed by the enzyme StAR. Together these proteins work to upregulate glucocorticoid synthesis at the discretion of the hypothalamus. As with the brain tissues analysed, IL-1β also plays a signalling role in the adrenal gland. The expression of the genes IL-1β, MC2R, P450SCC, StAR and GR was measured using qPCR. Results showed strain differences in basal gene expression of all genes measured except for GR. Significant upregulation of IL-1β in both strains in response to parasite infection demonstrated a significant role for IL-1b signalling during parasite infection. The Lewis strain had a significantly high basal level of MC2R expression which was downregulated to be comparable to that of Fischer. Expression of P450SCC was also higher in Lewis however basal StAR expression was significantly lower than that of Fischer. Both P450SCC and StAR showed strain differences in basal gene expression however the genes did not respond to parasite infection. The expression of GR was similar between strains and did not respond to parasite infection. The downregulation of MC2R seen in Lewis may be indicating that the system is under negative feedback and the system is geared against chronic HPA axis activation. Expression of IL-1β was similar to the upregulation seen in the brain tissue of the hippocampus and hypothalamus suggesting that IL-1β signalling is important in HPA axis regulation and may play a role in the negative feedback mechanism as with glucocorticoids.
Figure 3.11: Adrenal Gland IL-1β gene expression in Fischer and Lewis rats in response to H. bakerii. Fischer strain showed significantly high basal levels of IL-1β expression compared to Lewis. IL-1β expression was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

Upregulation of IL-1β due to parasite infection was observed in both strains. IL-1β was upregulated by 43% (p=0.001) in the Fischer strains whilst the Lewis strain saw a 63% (p=0.0003) upregulation of IL-1β in response to parasite infection. The two strains also differed significantly in terms of basal IL-1β expression with IL-1β expression being 53% (p=0.043) higher in the Fischer control group compared to the Lewis control group.
Figure 3.12: Adrenal Gland MC2R gene expression in Fischer and Lewis rats in response to *H. bakerii*. Lewis strain showed significantly higher basal levels of MC2R expression compared to Fischer. MC2R expression was downregulated in response to parasites in Lewis only. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

Significant downregulation of MC2R was observed in the Lewis strain only in response to parasite infection. MC2R was downregulated by 13% (p=0.027) in the Lewis strain only. Expression of MC2R was higher in Lewis strain, with MC2R transcripts being 22% (p=0.022) more abundant in control group rats from the Lewis strain than the Fischer strain controls.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure 3.13: Adrenal Gland P450 gene expression in Fischer and Lewis rats in response to *H. bakerii*. Lewis strain showed significantly higher basal levels of P450 expression compared to Fischer. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in P450 gene expression in response to parasite infection. The basal expression of P450 was higher in the Lewis strain than in the Fischer strain. The Lewis strain showed 27% (p=0.036) greater gene expression than that of Fischer.
Figure 3.14: Adrenal Gland StAR gene expression in Fischer and Lewis rats in response to *H. bakerii*. Fischer strain showed significantly higher basal levels of StAR expression compared to Lewis. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in StAR gene expression in response to parasite infection. The basal expression of StAR was higher in the Fischer strain than in the Lewis strain. The Fischer strain showed 56% (p=0.017) greater gene expression than that of Fischer.
Figure 3.15: Adrenal Gland GR gene expression in Fischer and Lewis rats in response to *H. bakerii*. There was no difference in GR expression between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (**p<0.001**).

There was no significant difference in adrenal gland expression of the GR of both strains in response to parasite infection. There was no significant difference between strains.
3.3.4 Small Intestine Gene Expression in Response to H. bakerii Infection

The small intestines are the site of physical infection where mucosal truma occurs. The burrowing of the larvae into the mucosa of the duodenum and proximal jejunum causes inflammation which generates the immune response. In rats the immune response is effective, causing significant tissue genesis resulting in the encasement of the larvae. This prevents the larvae from emerging into the small intestine to reproduce. Both pro-inflammatory cytokines and early onset pro-TH2 cytokines gene expression was measured using qPCR. Results showed significant IL-6 and IL-33 upregulation in response to parasite infection however IL-1β and TNFα gene expression remained constant or downregulated. The gene expression of GR was similar between strains and remained constant in response to parasitism. Interestingly, IL-25 expression was downregulated in Fischer whilst upregulated in Lewis in response to parasites. The results suggest that the initial classical inflammatory response, typified by high pro-inflammatory cytokine gene expression, was being scaled back whilst the TH2 response was beginning to intervene.
Figure 3.16: Small Intestine IL-1β gene expression in Fischer and Lewis rats in response to *H. bakerii*. Basal IL-1β expression was higher in Fischer compared to Lewis. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There were no significant changes in IL-1β gene expression in the small intestine in response to parasitic infection. The basal gene expression of IL-1β however was significantly higher in the Fischer strain than the Lewis strain. Basal expression of IL-1β was 357% (p=0.0001) higher in the Fischer strain than the Lewis strain.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure 3.17: Small Intestine IL-6 gene expression in Fischer and Lewis rats in response to *H. bakerii*. Basal levels of IL-6 expression was higher in the Fischer strain compared to the Lewis strain. Both strains demonstrated significant upregulation of IL-6 in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, ***=p<0.001).

The expression of IL-6 was upregulated in the small intestine tissue in response to parasite infection in both strains. In the Fischer strain, IL-6 was upregulated by 819% (p=0.01) and was similarly upregulated in the Lewis strain being upregulated by 1370% (p=0.0036) in response to parasite infection. The basal level of IL-6 expression also differed with expression being 73% (p=0.04) higher in Fischer than Lewis.
Figure 3.18: Small Intestine IL-25 gene expression in Fischer and Lewis rats in response to \textit{H. bakerii}. IL-25 expression was downregulated in Fischer, whilst upregulated in Lewis in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of IL-25 significantly differed in the small intestine tissue in response to parasitic infection. There was a significant change in gene expression of IL-25 observed in the Fischer group where IL-25 gene expression was downregulated by 31% (p=0.012). In comparison IL-25 expression in the Lewis strain was upregulated by 11% (p=0.05) in response to parasitic infection. There was no significant IL-25 expression difference between strains averaged over the experimental treatment groups.
Figure 3.19: Small Intestine IL-33 gene expression in Fischer and Lewis rats in response to \textit{H. bakerii}. The basal levels of IL-33 expression was higher in Fischer compared to Lewis. Expression of IL-33 was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of IL-33 was upregulated in the small intestine tissue in response to parasite infection in both strains. In the Fischer strain, IL-33 was upregulated by 31\% (p=0.009) and was similarly upregulated in the Lewis strain being upregulated by 60\% (p=0.011) in response to parasite infection. The basal level of IL-33 expression also differed with expression being 58\% (p=0.002) higher in Fischer than Lewis.
Figure 3.20: Small Intestine TNFα gene expression in Fischer and Lewis rats in response to *H. bakerii*. Basal expression levels of TNFα was higher in Fischer compared to Lewis. TNFα expression was downregulated in Fischer only in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was significant downregulation of TNFα in the small intestine tissue in response to parasitic infection. There was a 66% (p=0.02) decrease in gene expression in the Fischer strain in response to parasitic infection but not in the Lewis strain. The basal level of TNFα expression also differed with Fischer expression of TNFα being 105% higher than in Lewis.
Figure 3.21: Small Intestine GR gene expression in Fischer and Lewis rats in response to *H. bakeri*. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There were no significant differences in GR gene expression in the small intestine in response to parasitic infection. There were no significant strain differences.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. Fenner* (2012)

128
3.3.5 Mesenteric Lymph Node Gene Expression in Response to H. bakerii Infection

Being the major lymph node associated with the small intestine, the mesenteric lymph node plays an important role in the establishment and recruitment of T cells. The mesenteric lymph node (MLN) is an important site for TH2 cell activation with macrophages and other APCs draining from the infected small intestinal tissue. Interactions between APCs and TH2 cells results in type 2 cytokine production instrumental in establishing the TH2 response required for effective anti-parasite immune responses. The mesenteric lymph node is a crucial region for generating the antibody response, with IgG1 being produced during the acute and intermediate infection phase more so than the chronic phase [12]. Both type 1 and type 2 cytokine expression was measured by qPCR, as well as STAT4. FOXP3 expression was measured to determine the influence of T\text{reg} cells in the immune response. The TH2 immune response was well established at the MLN with the upregulation of type 2 cytokines at the expense of type 1 cytokines. This was most evident in the downregulation of IL-1β and the non-response of STAT4, IL-2 and TNFα, whilst IL-6, IL-10, and IL-13 were upregulated. There was no change in the expression of GR and FOXP3. The results suggest that the TH2 response is well established at the MLN.

The expression of STAT6 was beyond detectable limits for both strains.
Figure 3.22: Mesenteric Lymph Node IL-1β gene expression in Fischer and Lewis rats in response to *H. bakeri*. IL-1β expression was downregulated in both groups in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The gene expression of IL-1β in the mesenteric lymph node was downregulated in response to parasite infection. IL-1β gene expression was downregulated by 58% (p=0.0001) in the Fischer strain. Similarly, IL-1β gene expression was also downregulated in the Lewis strain by 40% (p=0.007). There was no significant difference in gene expression between strains.
Figure 3.23: Mesenteric Lymph Node IL-2 gene expression in Fischer and Lewis rats in response to *H. bakerii*. The Lewis strain had significantly higher level of IL-2 basal expression compared to Fischer. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in IL-2 gene expression in the mesenteric lymph node in response to parasitic infection. There was a strain difference in IL-2 gene expression with Lewis expressing 57% more IL-2 transcripts than Fischer.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*  
*N.O. FENNER (2012)*
There was significant upregulation of IL-6 in the mesenteric lymph node in response to parasitic infection. Expression of IL-6 in the Lewis strain was upregulated by 76% (p=0.003), whilst expression was similarly upregulated in the Fischer strain by 29% (p=0.05) in response to parasitic infection. There was also significant differences in basal IL-6 gene expression with Fischer having a 37% (p=0.006) increase in expression.
Figure 3. 25: Mesenteric Lymph Node IL-10 gene expression in Fischer and Lewis rats in response to *H. bakerii*. Basal levels of IL-10 expression was higher in Lewis compared to Fischer. IL-10 was upregulated in response to parasites in both strains. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The gene expression of IL-10 in the mesenteric lymph node was upregulated in both strains in response to parasitic infection. In the Fischer strain IL-10 expression was upregulated by 107% (p=0.04) whilst expression in the Lewis strain was upregulated by 217% (p=0.002) in response to parasitic infection. The basal gene expression of IL-10 significantly differed between strains with the Lewis strain being 1581% higher in basal gene expression.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

133
Figure 3.26: Mesenteric Lymph Node IL-13 gene expression in Fischer and Lewis rats in response to *H. bakerii*. IL-13 expression was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of IL-13 in the mesenteric lymph node was significantly upregulated in both strains in response to parasitic infection. The gene expression of IL-13 was upregulated by 7055% (p=0.01) in the Fischer strain while in the Lewis strain IL-13 expression was upregulated by 2622% (0.008) in response to parasitic infection. There was no significant difference in basal IL-13 between strains.
Figure 3.27: Mesenteric Lymph Node STAT4 gene expression in Fischer and Lewis rats in response to *H. bakerii*. Basal levels of STAT4 expression was higher in Lewis compared to Fischer. STAT4 expression was downregulated in response to parasites in both strains. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (**=p<0.01, ***=p<0.001).

The gene expression of STAT4 in the mesenteric lymph node was significantly downregulated in both strains in response to parasitic infection. The gene expression of STAT4 was downregulated by 110% (p=0.03) in the Fischer strain while in the Lewis strain STAT4 expression was downregulated by 142% (p=0.0001) in response to parasitic infection. The basal gene expression of STAT4 differed significantly with STAT4 transcripts being 3244% more abundant in the Lewis strain than in the Fischer strain.
Figure 3.28: Mesenteric Lymph Node FOXP3 gene expression in Fischer and Lewis rats in response to *H. bakerii*. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in FOXP3 gene expression in the mesenteric lymph node. There was no difference in FOXP3 gene expression between strains.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*  
N.O. FENNER (2012)
Figure 3.29: Mesenteric Lymph Node GR gene expression in Fischer and Lewis rats in response to *H. bakerii*. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in GR gene expression in the mesenteric lymph node. There was no difference in GR gene expression between strains.
Figure 3.30: Mesenteric Lymph Node TNFα gene expression in Fischer and Lewis rats in response to *H. bakerii*. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in TNFα gene expression in the mesenteric lymph node. There was no difference in TNFα gene expression between strains.
There was no significant change in IFNγ gene expression in the mesenteric lymph node. There was no difference in IFNγ gene expression between strains. The expression of IFNγ was beyond detectable limits.
3.3.6 Spleen Gene Expression in Response to H. bakerii Infection

Parasite infection involve the spleen in two ways. Firstly, parasites may directly affect the spleen through larval colonisation or indirectly through the filtering out of parasite antigens. *H. bakerii* infection does not have any direct effects on the spleen in terms of tissue infection by the larval stage, however circulating antigens may be monitored by the spleen. It was hypothesised that the spleen may come into contact with parasite specific antigens through circulating APCs and blood-bourne antigens, initiating an immune response. This immune response may be generated through the presentation of the antigens by APCs to resident lymphocytes. The spleen may act as a tissue for the generation of systemic TH2 cytokines and pro-inflammatory cytokines [33]. These cytokines may also be monitored by the HPA axis through the negative feedback mechanism. The same panel of gene expressions where measured in the spleen as measured in the MLN. Gene expression of type 1 and type 2 cytokines in the spleen was measured to determine the cytokine levels that may have been released into systemic circulation. Results suggested that even though the TH2 response appeared dominant it was less obvious, with the downregulation of IL-6 and the stability of expression of IL-10. Interestingly, IL-1β was upregulated in the spleen. This opposed that data found in the MLN. Overall, the results showed that the response at the spleen was weaker than the MLN. As with the MLN, the spleen was high in IL-13 expression.

The expression of IL-2 and STAT6 was beyond delectable limits in both strains.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?  
* N.O. FENNER (2012)  
140
Figure 3.32: Spleen GR gene expression in Fischer and Lewis rats in response to *H. bakerii*. The basal level of GR expression was higher in the Lewis strain compared to the Fischer. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in GR expression in the spleen in response to parasitic infection in either strain. The basal level of gene expression of the GR differed significantly with the Lewis strain having 72% (p=0.01) higher GR expression than the Fischer strain.
The expression of IFNγ in the spleen was downregulated in the Lewis strain by 61% \( (p=0.02) \) in response to parasitic infection. There was no significant change in IFNγ gene expression in response to parasitic infection in the Fischer strain. There was no significant difference in the basal level of IFNγ gene expression between strains.
Figure 3.34: Spleen IL-1β gene expression in Fischer and Lewis rats in response to *H. bakeri*. Basal levels of IL-1β expression was higher in the Lewis strain compared to the Fischer. IL-1β expression was upregulated in the Fischer strain only. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of IL-1β in the spleen was upregulated in the Fischer strain by 38% (p=0.03) in response to parasitic infection. There was no significant change in IFNγ gene expression in response to parasitic infection in the Lewis strain. The basal levels of IL-1β gene expression differed between strains with the Lewis strain having 49% (p=0.02) higher basal gene expression than Fischer.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

N.O. FENNER (2012)
Figure 3.35: Spleen IL-6 gene expression in Fischer and Lewis rats in response to *H. bakerii*. Basal levels of IL-6 expression was higher in Fischer compared to Lewis. IL-6 expression was downregulated in response to parasites in both strains. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of IL-6 in the spleen was downregulated in both strains in response to parasitic infection. IL-6 gene expression in the Fischer strain was downregulated by 85% (p=0.003), while IL-6 expression in the Lewis strain was downregulated by 69% (p=0.001) in response to parasitic infection. The basal levels of IL-6 gene expression differed between strains with the Fischer strain having 74% (p=0.008) higher basal gene expression than Lewis.
Figure 3.36: Spleen IL-13 gene expression in Fischer and Lewis rats in response to *H. bakerii*. IL-13 expression was upregulated in both strains in response to parasites. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (**p<0.01**).

The expression of IL-13 in the spleen was upregulated in both strains in response to parasitic infection. IL-13 gene expression in the Fischer strain was upregulated by 479% (p=0.004), while IL-13 expression in the Lewis strain was upregulated by 639% (p=0.001) in response to parasitic infection. There was no significant difference in basal IL-13 gene expression between strains.
Figure 3.37: Spleen STAT4 gene expression in Fischer and Lewis rats in response to *H. bakerii*. STAT4 expression was downregulated in response to parasites in the Lewis strain only. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

The expression of STAT4 in the spleen was downregulated in the Lewis strain by 61% (p=0.03) in response to parasitic infection. There was no significant change in IFNγ gene expression in response to parasitic infection in the Fischer strain. There was no significant difference in the basal level of IFNγ gene expression between strains.
Figure 3.38: Spleen FOXP3 gene expression in Fischer and Lewis rats in response to *H. bakerii*. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in FOXP3 gene expression in the spleen. There was no difference in FOXP3 gene expression between strains.
Figure 3.39: Spleen IL-10 gene expression in Fischer and Lewis rats in response to *H. bakeri*. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in IL-10 gene expression in the spleen. There was no difference in IL-10 gene expression between strains.
Figure 3.40: Spleen TNFα gene expression in Fischer and Lewis rats in response to \textit{H. bakerii}. There was no significant differences between groups. Yellow bar denotes F-C (Fischer Control), Orange bar denotes F-P (Fischer Parasite), Green bar denotes L-C (Lewis Control), and Blue bar denotes L-P (Lewis Parasite). Lines group significantly different treatment groups, with significance being signified by stars (*=p<0.05, **=p<0.01, and ***=p<0.001).

There was no significant change in TNFα gene expression in the spleen. There was no difference in TNFα gene expression between strains.
3.3.7 Corticosterone RIA

Corticosterone is the end product of activation of the HPA axis. Corticosterone as with other glucocorticoids downregulates the inflammatory response as well as liberating resources for the immune response. The level of plasma corticosterone differed only at a single timepoint in the Fischer rat strain in response to parasitic infection. On day 6 of parasite infection the Fischer rat strain showed a significant downregulation of plasma corticosterone, where plasma corticosterone was reduced by 113.8mg/mL (p=0.03) in the parasite infected group compared to the parasite free control. This equated to a 84% reduction in plasma corticosterone.

There were a number of timepoints where there was a significant difference in plasma corticosterone concentrations between strains. At day -3 plasma corticosterone levels in the Lewis were 219mg/mL (p=0.04) less than the Fischer, and at day -1 plasma corticosterone levels in the Lewis were

Figure 3.41: Corticosterone response of Fischer and Lewis rats to *H. bakerii*.
145mg/mL (p=0.04) less than the Fischer. This equated to Lewis corticosterone levels being 109% and 68% less than Fischer at timepoints -3 and -1 respectively.

### 3.3.8 Liver Analysis

Permanently elevated glucocorticoid levels causes fat to be deposited in livers in a process known as hepatic steatosis. This is evident in human conditions such as Cushing’s disease [34]. However, glucocorticoids are also important in liberating immune response resources such as blood glucose and protein catabolism. The weights and lipid contents of livers was measured to ascertain the effects of parasitism and HPA axis activity on liver fat content. The results demonstrated that the Lewis rat strain had larger livers in terms of overall weight at kill as well as a greater liver fat content than Fischer. This is opposed the results that Lewis had lower plasma corticosterone levels than Fischer. The results suggest that glucocorticoid levels may play a role in regulating the fat content of livers in parasitised rats. From the results it was evident that in the Lewis strain parasitism reduced liver fat content significantly, but was not seen in the Fischer. Interestingly, there is seems to be a negative correlation between liver weights & fat content and plasma corticosterone levels. This suggests that decreased corticosterone levels of the Lewis may be involved in fat deposition in the liver or the higher corticosterone levels of the Fischer may assist in liberating fats/block fat deposition in the liver. The results also suggest that elevated glucocorticoid levels in Cushing’s disease are not relevant to the high levels seen in the Fischer strain.
Liver Weights at Kill

Figure 3.42: Liver weights at kill. Weights of livers at the time of dissection. Livers were removed and the entire liver weighed. There was no significant differences between treatment groups, with parasite infection having no effect on liver weight. There was a significant difference between strains, with Lewis having livers with a greater mass than Fischer.

There were no significant effects of treatment; however there was a significant difference in the weight of livers between strains. The Lewis strain on average had a larger liver than that of the Fischer strain. The Lewis liver was 2.19g (p=0.00002) heavier than the Fischer which equates to a 19.5% heavier liver.
Liver Fat Content

**Figure 3.43: Percentage liver fat.** Total extractable liver fats as a percentage of wet liver weight. Liver fat content was reduced significantly in the Lewis treatment group but this was not observed in the Fischer treatment group. There were no significant differences between strains in terms of liver fat content.

There was a significant decrease in the fat content of Lewis strain livers in response to parasite infection. This observation was not found in the Fischer strain. The fat content in the Lewis strain livers was decreased by 44% (p=0.0001) from 17.4% to 12.1%. The basal fat content also differed between strains with the Lewis strain having 33.7% (p=0.02) greater liver fat content than Fischer.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

153
3.3.9 Body Temperature

The hypothalamus monitors the internal milieu of a mammal where disruptions to the internal milieu are counteracted and regulated at the discretion of the hypothalamus. *H. bakerii* parasitism was hypothesised to cause significant inflammation in the small intestinal tissue which would be monitored by the hypothalamus triggering HPA axis activation. There was however no significant change in rectal temperature in response to parasitism suggesting that the inflammatory effects of parasitism may not be significant enough to cause physiological changes in body temperature. This result may help to explain the lack of glucocorticoid response seen in plasma.

![Figure 3.44: Changes in Body Temperature.](image)

There was no significant change in rectal temperature in response to parasitic infection in either strain. There was no difference in basal rectal temperature between strains.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
3.3.10 Body Weight

The age matched Fischer and Lewis rat strains differed in body weight during the experiment. The Lewis strain showed a higher body weight compared to Fischer. The Lewis strain was on average had a 23.4% greater body weight compared to Fischer over the course of the experiment. The final body weights at euthanisation supported the body weight measurements taken over the course of the experiment. Other results also showed a greater significant difference in liver weight & liver fat composition coupled with the fact that the Lewis strain has lower plasma corticosterone levels. Results also showed that body weights of both strains were no affected by parasite burden. These results support other results suggesting that reduced corticosterone levels seen in the Lewis may affect metabolism, resulting in weight gains. However, weight gains over the course of the experiment did not differ between strains nor affected by parasite burden. The Lewis strain had a significantly higher rate of food consumption but this did not translate into differing weight gains. Therefore differences in body weights seen over the course of the experiment were not due to weight gains during the course of the experiment or as an effect of parasitism, but rather due to early growth possibly affected metabolically by the respective HPA axis activity of the two strains.
There was no effect of parasite infection on body weight during the experimental timecourse. The Lewis strain was significantly heavier in weight during the entirety of the experiment compared to Fischer, with an average 23.4% difference in body weight.
Figure 3.46: Average Body Weight at Kill.

There was no difference in the body weight at the time of kill in response to parasitic infection. The body weight of the two strains differed significantly at the time of kill with the Lewis strain being 56.3g heavier in weight than the Fischer strain equating to 24% difference in body weight.
Figure 3.47: Average Weight Gains during Experiment.

Over the timecourse of the experiment there was no significant change in weight in response to parasitic infection. The weight change did not differ between strains.
3.3.11 Food Consumption

There was no significant difference in food consumption as a result of parasitic infection. On average, the Lewis strain consumed 1.5g more feed per rat per day than the Fischer strain, which equated to a 18.5% higher feed consumption.

Figure 3.48: Average Food Intake per day.
3.3.12 qPCR Analysis of Parasite DNA in Small Intestinal Tissue

*H. bakerii* has a life cycle stage which involves sub mucosal larval maturation in both the rat and mouse. In the rat, the sub mucosal stage is terminal as the larvae is encapsulated in the tissue. Therefore, qPCR may be used to semi-quantify worm burden, and to validate the absence of parasites in the control groups. DNA was extracted from small intestinal tissue stored in ethanol. Primers were used to amplify DNA amplicons of both host and parasite DNA. The results demonstrated the validity of the experiment with the controls being absent of parasite DNA, whilst the treatment groups were positive for parasite DNA. Furthermore, there was no significant difference between host and parasite DNA per treatment group suggesting that both strains received similar doses. This result also suggests that neither strain differed in it’s ability to prevent sub mucosal invasion.

3.49: qPCR analysis of parasite DNA in small intestinal tissue. The blue bars refer to host DNA (REF). The red bars refer to parasite DNA (ITS2). Since *H. bakerii* is encapsulated in the small intestinal tissue a measure of parasite DNA is an indication of parasite burden.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Both strains showed positive qPCR results for parasite (ITS2) DNA in parasitised small intestinal tissue. Similarly, parasite DNA was absent from control groups in both strains. The ratio of host:parasite DNA was not significantly different between groups. Therefore there was no contamination of the control group with parasites, demonstrating the infection methodology was effective.

3.3.13 Faecal Egg Counts
Both strains received consistent doses of parasites and there was no difference in prevention of parasite sub mucosal invasion. In both the Lewis and Fischer parasitised rats there were no positive FECs. There were no positive FECs in the Lewis or Fischer controls. This suggests that neither strain was susceptible to parasite infection with both strains mounting an effective immune response to *H. bakerii* at the sub mucosal stage preventing the development of larvae into sexually mature adults.
3.4 Discussion

The infection of Fischer and Lewis rat strains by *H. bakerii* infection showed that both strains were resistant to parasite establishment, blocking infection at the submucosal stage. This is consistent with pioneering research into *H. polygyrus* infection in the white rat where glucocorticoid treatment was able to reverse the resistance of the rat to *H. bakerii*, through the inhibition of the formation of the encapsulating connective tissue granuloma hypothesised to be driven by inflammatory processes (see figure 3.2) [13, 14]. The experiment aimed to analyse the effects of the HPA axis on parasite immune response by utilising rats strains with differing HPA axis activity, although resistance to parasite infection did not differ between strains.

The HPA Axis

The HPA could potentially have a major influence over the immune response to *H. bakerii* via its control of the neuroendocrine system (signalling between the hypothalamus, pituitary and adrenal glands). The experiment described in this chapter analysed the hypothalamus and adrenal glands, as well as the extra-HPA axis tissue of the hippocampus. The qPCR results obtained from the HPA axis tissues suggest the HPA axis, at the time of sampling, was possibly being influenced by the negative feedback mechanism involved in regulating HPA axis activity, evident in the downregulation of MC2R and the lack of gene expression change in P450 and StAR. Negative feedback may also explain the absence of a plasma corticosterone response to parasite infection. The upregulation of CRH was both strain and tissue specific, with CRH upregulation in Fischer in the hippocampus, whilst the upregulation of CRH in Lewis rats was in the hypothalamus. Also, the Fischer strain showed upregulation of GR in the hypothalamus only. The GR was unchanged in the hippocampus and the adrenal gland. Interestingly, the upregulation of the pro-inflammatory cytokine IL-1β in response to parasite infection was unexpected since there was an absence of glucocorticoid synthesis. However, expression of IL-1β in brain tissue is more pronounced in the absence of glucocorticoid synthesis [28]. Both
strains saw upregulation of IL-1β in the hippocampus, hypothalamus and the adrenal gland. The upregulation of brain synthesised IL-1β has been documented in various models of stress [27, 28, 35, 36]. It is thought that IL-1, in its many forms, may activate the HPA axis in response to immunological stressors such as endotoxins [37, 38].

It is believed that the results presented in this chapter may be the first documented evidence of brain transcribed IL-1β in rats responding to parasitism. Also of interest was the observation that the Lewis strain, whilst having a similar IL-1β response in the hippocampus as Fischer there was a significant lack of CRH expression. However, both strains had similar IL-1β expression in the hypothalamus but CRH expression did not significantly change in the Fischer strain, unlike Lewis. Whilst IL-1β expression was upregulated in both strains in both the hippocampus and the hypothalamus this did not correlate with CRH expression suggesting that CRH expression and not IL-1β expression may be involved in the neuroendocrine differences between the two strains.

Upregulation of IL-1R1 was also seen for both strains in the hypothalamus only. The main receptor (MC2R) involved in HPA-axis signalling in the adrenal gland was downregulated in the Lewis strain only.

The results suggest that the lack of corticosterone synthesis may loosely correlate with a lack of gene expression changes in the HPA axis tissue, especially the adrenal gland where downregulation of MC2R was evident. IL-1β expression in the HPA axis tissue suggests that IL-1β has a direct role in regulating HPA axis activity in parasitised rats. IL-1β and its receptor may be involved in the activation of the HPA axis and may be a mediator of hyperactivity [27, 28, 35, 36, 39, 40]. Hyperactivity of the HPA axis was not observed in terms of corticosterone output however, suggesting that IL-1β in this incidence may have another role. To investigate the role of IL-1β in regulating the HPA axis in response to parasite infection, further experiments must include the role of other key players such as IL-1α and Interleukin-1 receptor agonist (IL-1Ra). The role of IL-1β and IL-1R1 without examining the role of IL-1α and IL-1Ra would be to

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

163
make conclusions out of context, since neither of these proteins where examined in this experiment. Of importance would be the agonist IL-1Ra which may play a role in regulating IL-1β activity. Since both proteins bind to the same receptor conclusions made about the role of IL-1β would need to be in the context of IL-1Ra expression. However, it is evident that IL-1β and IL-1R1 gene expression does change in response to parasite infection and so may be involved in the neuroendocrine response to infection. Upregulation of hypothalamus GR gene expression in the Fischer strain suggests that the hypothalamus is adapting to chronic glucocorticoid levels and HPA axis hyperactivity which is consistent with elevated IL-1β [35]. Similarly, downregulation of adrenal MC2R in the Lewis rats suggests negative feedback in response to hyperactivity. This is contradicted by the upregulation of CRH in the hypothalamus. To examine the state of HPA axis activity, the analysis needs to be taken to the next stage with measurements on the protein levels of the genes in question. Although changes in gene expression were observed, post-transcriptional regulation may be changing the actual protein levels of the genes, however this was not measured and presents an area for future investigation. Since the activity of the HPA axis is crucial for maintenance of homeostasis, it is conceivable that gene transcription and translation would be highly regulated.

**Small Intestine**

The small intestine is the site of infection for *H. bakerii*, and significant gene expression changes were observed between rat strains in this tissue. In the absence of infection, Fischer had higher basal gene expression of both IL-1β and TNFα whilst IL-6 and IL-33 were upregulated in response to parasite infection in both strains. Interestingly, IL-25 expression was downregulated in Fischer and upregulated in Lewis in response to parasite infection. These results demonstrate significant phenotypic differences at the site of infection, between the rat strains where Fischer demonstrated TH1 profile tendencies, and Lewis was more markedly TH2 biased. This is typified by the higher basal
levels of pro-inflammatory cytokines and the downregulation of IL-25 in Fischer rats in response to infection. With lower levels of pro-inflammatory cytokine gene expression in controls and the upregulation of IL-25 in response to parasite infection, Lewis rats might have been mounting a more TH2 biassed response at the site of infection. Despite the differences, parasite infection in both rat strains stimulated upregulation of both IL-6 and IL-33 suggesting that H. bakerii infection initiates a TH2 response in both strains. Therefore, qPCR results at the site of infection suggests that the Lewis strain establishes a stronger TH2 response than the Fischer strain, typified by the upregulation of IL-25 and the lower basal expression of pro-inflammatory cytokines, and the upregulation of IL-33 and IL-6 [41, 42]. This suggests that basal gene expression, possibly regulated by basal HPA axis activity, may be important in shaping the immune response at the site of infection.

Mesenteric Lymph Node
The MLN is the main draining lymph node associated with the small intestine. In the MLN, the rat strains had similar qPCR profiles in response to parasite infection. Both strains demonstrated significant upregulation of the central TH2 related cytokine IL-13 whilst actively downregulating the pro-inflammatory cytokine IL-1β, and with little evidence of TNFα or IFNγ expression. Genes associated with the TH1 response where either downregulated or unchanged in response to H. bakerii. Like the small intestines, the expression of the GR was unchanged in response to parasite infection. Important TH2 related genes IL-6, IL-10 and IL-13 were significantly upregulated in response to parasite infection whilst IL-1β and the TH1 related transcription factor STAT-4 was downregulated. Interestingly, gene expression of IL-2, IFNγ and TNFα was unchanged in response to parasite infection. There were significant differences in the levels of basal gene expression of certain genes. The gene expression of pro-TH1 cytokines IL-2 and the TH1-related transcription factor STAT-4 were all significantly higher in the Lewis strain compared to the Fischer. With the observation that the pro-TH2 cytokine IL-6 was expressed higher in the Fischer

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?
N.O. FENNER (2012)
strain than the Lewis, these data suggest a more marked TH2 bias for Fischer than Lewis. However, the pro-TH2 cytokine IL-10 was found to be expressed at a higher basal level in the Lewis than Fischer strain, and the results obtained showed that both strains responded similarly to parasite infection. On balance, there may be a slight TH1 bias for Lewis with the basal cytokine environment of the MLN geared towards TH1 responses in this strain. This suggests that low corticosterone abundance in Lewis plasma may help to establish a TH1 environment at a basal level. However, there was no change in GR suggesting that this may be regulated by another means. Over all though, in response to parasites in the small intestine, gene expression analysis of the MLN demonstrated a classical TH2 response in both rat strains. This shows that any biases between TH1 and TH2 shown by Lewis or Fischer rats had little effect on the parasite response which remained TH2 biassed in both strains.

**Spleen**

The MLN is the main draining lymph node associated with the small intestine, but the spleen is important for the detection of antigens carried within the blood. Antigens from the submucosal stage of *H. bakerii* infection could enter the circulatory system directly, or be carried by antigen presenting cells into the circulatory system and may subsequently be detected by cells residing in the spleen. As with the small intestine the Lewis strain demonstrated a tendency towards TH2 responses, whilst the Fischer strain showed conflicting results. The pro-TH1 associated gene IL-1β was upregulated in Fischer rats and STAT4 and IFNγ were downregulated in the Lewis rats, consistent with Fischer being more TH1 biassed than Lewis. However, the expression of IL-13 was upregulated while IL-6 was downregulated by parasite infection in both strains, showing that there were dominant gene expression pattern changes that did not differ between rat strains. Gene expression in uninfected rats also differed significantly between strains. IL-1β, which was upregulated in the Fischer strain, had a higher basal expression in the Lewis strain compared to the Fischer. Similarly, IL-6 was expressed at a higher level in the uninfected Fischer rats.
when compared to the uninfected Lewis animals. Conversely, GR was expressed at a higher level in the uninfected Lewis rats than the uninfected Fischer rats. The results obtained demonstrated that the spleen may be involved in parasite infections. It is not possible to distinguish whether circulating antigens, circulating activated macrophages or both are important in the spleen’s response to parasitism of the small intestine. Similarly, the presence of IL-13 in the spleen suggests activated T-lymphocytes but it is not certain whether these lymphocytes were activated at the spleen or were circulating activated lymphocytes from the site of infection.

![Figure 3.50: The spleen and the mesenteric lymph node may have similar roles in the immune response to *H. bakerii*. Lymphocyte activation may occur in two ways. Antigens originating at the site of infection, the small intestine, are carried to the spleen in the bloodstream while antigens are carried to the MLN via lymphatic fluid. Resident macrophages engulf the free antigens via phagocytosis. Antigen presentation occurs, activating lymphocytes. Similarly, macrophages at the site of infection phagocytose parasite antigens before migrating to the spleen or MLN via the blood stream or lymphatic fluid respectively, where activation of lymphocytes occur via antigen presentation. These activated lymphocytes then travel to the site of infection via the blood stream or lymphatic fluid.](image)

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

167
Physiological Changes

The absence of physiological changes in rats parasitised with *H. bakerii* suggests that the immunological impact on the rats was quite mild. The one interesting result from analysing physiological changes in parasitised rats showed that the liver fat composition of the Lewis rat was reduced in response to parasite infection. This result was not seen in the Fischer rat, suggesting that lower basal corticosterone levels in the Lewis may assist in liberating fat reserves in time of infection. This would be particular importance in response to a heavy parasite burden where the parasites would be impacting on energy intact from food consumption through reduced nutrient absorption at the site of infection, the gastrointestinal tract. The Lewis rat strain had a larger body mass although the body mass of neither strain was affected by parasite infection. The Lewis strain also had an increased level of food consumption, which may contribute to the larger body mass. Therefore, it is evident that the worm burden used in this experiment may not have been larger enough to insight HPA axis activation, nor any changes in physiology.

Further Research & Experimental Limitations

One aspect of the immune response to *H. bakerii* in rats that was not considered was the involvement of B cells in both regulating the immune response through cytokine expression and contributing to the immune response through antibody production. Gene expression results from the MLN showed significant upregulation of IL-10. We do not know the origin of the IL-10 mRNA detected, but it may have been from B cells, as well as activated T cells [10].

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure 3.51: Hypothesised origins of MLN cytokine upregulation. The upregulation of IL-6, IL-10 and IL-13 was observed in the MLN. Both activated APCs and parasite antigens drain back to the MLN. Here at the MLN, APCs present antigen to naïve T cells initiating the TH2 response. Activated macrophages are expressing IL-6. Activated TH2 cells begin expressing IL-10 and IL-13. B cells recognise parasite antigens and also present antigens to T cells. Activated B cells express IL-10, which further amplifies the TH2 response.
Conclusions

It was evident that *H. bakerii* infection did not induce major changes in HPA axis expression or corticosterone synthesis. Despite gene expression differences in HPA axis tissues between strains, the expression of IL-13 was notable in both the spleen and MLN in both strains in response to *H. bakerii* infection. Similarly, the upregulation of IL-6 and IL-10 in the MLN and IL-6 and IL-33 in the small intestine also demonstrate the strong TH2 response generated. However, upregulation and the lack of downregulation of certain TH1 genes provided evidence suggesting that the Fischer strain may be slightly TH1 biassed but with no observable gross effect on infection outcome. It is likely that the slightly higher plasma corticosterone concentration observed in Fischer rats is correlated with the differences observed in gene expression relative to Lewis rats, however the level is not sufficiently high to cause an ineffective immune response against *H. bakerii* such as that observed when high levels of exogenous glucocorticoids are administered [13, 14]. The experiment demonstrated a possible distinct role for the IL-1β in the neuroendocrine response to parasite infection. Although further investigation of this response was not conducted it is hypothesised that IL-1β in this instance in involved in neuroendocrine signalling, however HPA axis activation was not observed, suggesting that the system may in a state of negative regulation. Further investigation is needed in this area and future experimentation would concentrate on IL-1β signalling, taking into account IL-1α, IL-1 receptors and IL-1 agonists which were not examined.
3.5 References


CHAPTER 4: Co-culture of rat gastrointestinal cell lines with *Heligmosomoides bakerii* induces changes in gene expression: a model for gastrointestinal nematode infection *in vitro*

4.1 Introduction

The TH2 mediated immune response is crucial for an effective defence against parasites and has been implicated in inflammatory allergic reactions. The TH2 immune response is classically identified by the synthesis of type 2 cytokines such as IL-4, IL-5, IL-10 and IL-13 by CD4^+^ T-lymphocytes. The TH2 response typically results in B cell IgE production, and eosinophil stimulation. Initiation of the TH2 response however is poorly understood *in vivo*. Emerging research currently suggests that one mode of TH2 initiation may involve the production of IL-25 and IL-33 from affected cells, such as lung smooth muscle cells in asthmatic allergic reactions, or gastrointestinal colonocytes in ulcerative colitis [1-3]. Intestinal epithelial cells are an important target cell due to their location at the site of infection and as a first line of defence in the initial immune response. Intestinal epithelial cells are important primary immune cells being receptive to cytokines and being able to express chemokines and receptors [4, 5]

4.1.1 The role of Interleukin-33 and Interleukin-25 in gastrointestinal immune responses

The presence of IL-25 and IL-33 in the cellular environment may dictate whether the immune response is geared towards a TH2 type response [6]. Stimulation of epithelial cells by either parasites or allergenic factors leads to the secretion of IL-25 and IL-33 [6-8]. Similarly, cell damage or necrosis also leads to the release of intracellular IL-33 [7]. These cytokines stimulate non-B non-T cells, which may be a single population or a mixed population of natural

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
helper cells, nuocytes and multipotent precursor (MMP) cells, as well as basophils and dendritic cells [9]. Stimulation results in cellular differentiation and type 2 cytokine synthesis which in turn initiates TH2 differentiation, B cell proliferation and activation of parasitic-expulsion mechanisms [10-15].

IL-33 is a relatively recently discovered member of the IL-1 cytokine family and is structurally similar to IL-18 [16-18]. The IL-33 receptor complex, ST2 is associated with TH2 and mast cells [19]. IL-33 has been implicated in TH2 inflammatory responses. As well as the above mentioned cellular types, IL-33 also acts on eosinophils and mast cells [16, 20], both cell types implicated in allergenic responses and parasite expulsion [11, 12, 15, 21-24]. Activation of eosinophils by IL-33 is characterised by a marked increase in superoxide anion production and degranulation, which is comparable to IL-5 stimulation in vitro [16]. In vivo, the over-expression of IL-33 in mice resulted in the generation of pulmonary inflammation, suggesting that IL-33 is a significant signaler or mediator of inflammatory responses in the lung [2, 25]. Correspondingly, treatment with anti-IL-33 in vivo is able to diminish inflammatory effects resulting in a reduction of TH2 characteristics such as decreased eosinophil and lymphocyte populations, reduced IgE and reduced type 2 cytokines in the bronchoalveolar lavage fluids [1].
Gastrointestinal epithelial cells detect the presence of gastrointestinal nematodes leading to secretion of IL-33 and IL-25 or release IL-33 through cell damage or necrosis. These cytokines interact with dendritic cells, basophils and non-T, non-B cells. Dendritic cells present antigen via MHC class II without IL-12 secretion, similarly basophils present antigen also secreting IL-4, TSLP and IL-25, whilst non-T, non-B cells present antigen and secrete IL-4, IL-5 and IL-13. The presentation of antigen and cytokine secretion is received by TH2 cells upregulating the TH2 response in the form of type 2 cytokines expression B1 cell proliferation and activation of anti-helminthic effector pathways.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
4.1.2 The Role of Basophils & Dendritic cells

The TH1 mediated immune response to bacterial and viral antigens is well characterised and studied. It involves antigen presentation by APC via TCR of naïve CD4+ T lymphocytes [27] and the expression of the transcription factor T-bet, IL12 and STAT4 to promote TH1 differentiation and the production of type 1 cytokines [27]. The TH2 mediated immune response differs from this classical APC-T cell pathway. Dendritic cells are known to be poor producers of IL-4, the primary cytokine in the differentiation of naïve CD4+ cells to TH2 cells and the key driver of the TH2 response. Where it was previously thought that a number of accessory cells were involved including eosinophils, basophils, and Nature Killer cells (CD16+), it is now evident that basophils are a central cell type in antigen presentation in TH2 differentiation [16-18]. Three papers recently published in Nature immunology demonstrate that basophils are functionally crucial in the establishment of the TH2 response, whereby antigen presentation and IL-4 synthesis is key [28-30]. Dendritic cells play a major role in the establishment of the TH1 response, however are not required for the establishment of the TH2 response. When involved, dendritic cells are not exclusively sufficient to generate the TH2 response [18].

4.1.3 Cytokines: effects on gastrointestinal epithelial cells

Cytokines are essential in the regulation of the immune response. As well as acting on immune cells, cytokines also play a role in regulating other cell types such as epithelial cells [19-21]. Proliferation of IEC6 cells is initiated through the reception of IL-4, a major type 2 cytokine. Interestingly, IL-4 reception does not effect the secretion of IL-6 [33]. Therefore, IL-4 increases IEC6 cell proliferation whilst not effecting the amount of IL-6 being secreted resulting in a continuous pro-inflammatory signal being generated [33]. IL-4 and IL-13 also contribute to non-T cell immune responses against gastrointestinal parasites. These cytokines induce the differentiation of intestinal epithelial cells to goblet cells [34]. These goblet cells are then responsible for the secretion of RELMβ, a resistin-like molecule, essential for IL-4 mediated parasite expulsion. RELMβ is
an important anti-parasite molecule affecting the feeding capabilities of *H. polygyrus*. Cytokines also regulate effector responses in nematode effected cells such as the generation of mucin, important in the immune response to gastrointestinal nematodes. IL-4 and IL-13 directly regulate sulphotransferase HS3ST1 expression in IEC6 cells in the absence of ES. However, whilst co-incubation of ES products and IL-13 initiated some MUC2 gene expression, incubation with live *N. brasiliensis* larvae initiated much more MUC2 gene expression [35]. Therefore, some mucin-related genes are directly regulated by cytokines whilst others may be expressed more directly in response to nematode-derived molecules (PAMPs) binding to toll receptors or other pathogen recognition receptors. As well as activating type 2 cytokine production, IL-33 also drives pathological changes in the gut mucosa including changes in crypt length and epithelial cell proliferation [25].

4.1.4 Glucocorticoids: effects on gastrointestinal epithelial cells

Glucocorticoids affect many cell types including gastrointestinal epithelial cells [36]. Corticosterone is vital in the development and maturation of the rat intestinal tract including digestive enzyme appearance, expression of gastrin receptors and pinocytosis [37]. Studies have shown that the synthetic glucocorticoid DEX, can reduce inflammatory responses in IEC6 cells by inhibiting IL-1β-induced NFκB gene transcription [5]. However, this was not seen in Caco-2 cells where overexpression of GR in the absence of DEX resulted in NFκB inhibition. Another glucocorticoid, hydrocortisone, has also been shown to directly effect mucosal cells resulting in growth arrest, tight junction formation, changes to microvilli, and changes to ER and golgi networks. In response to hydrocortisone, cell growth plateaued in a dose dependant manner in cultures where media was not changed (for the 12 day culture period). Other results from the same study suggested that the glucocorticoid-mediated inhibition of cell growth was not permanent but was reversible dependant on cell density. The growth inhibitory properties of glucocorticoids may be repressed by Ras transformation through

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
jun and fos expression [38].

4.1.5 Experimental Design
In chapter 2 the effect of basal HPA axis activity was examined in the context of an immune response mounted against simple antigen/adjuvants in rats. Although the results were not conclusive the experiment supported the notion that Lewis rats are inflammation susceptible whilst Fischer rats are inflammation resistant. Furthermore, the Fischer strain showed a tendency towards a TH2 bias. Chapter 3 built on these results by examining the primary response of the two strains to the parasitic nematode *H. bakerii*. Results suggested that the basal level of HPA axis activity may influence the immune response generated. The Lewis strain, which has a lower plasma corticosterone concentration than Fischer was shown to generate a more robust TH2 environment. However, both strains were resistant to parasite establishment and both generated an internal environment that was TH2 biased in terms of cytokine expression. This chapter will examine both the glucocorticoid effects and the parasite effects on the host interface at a cellular level, by analysing cell growth and gene expression.

4.1.6 Aims
The experiments described in this chapter aim to replicate the host-parasite interface *in vitro*, in a cell and parasite co-culture system. Rat gastrointestinal cell lines will be co-cultured with parasitic larvae and the effects of parasites on those cells measured. Experiments aimed at examining the effect of glucocorticoids on basal cell growth and gene expression and of cell growth and gene expression in response to parasites are also described.

4.1.7 Hypothesis
Parasitic infections are known to incite a TH2 response *in vivo*. The hypothesis that *in vitro* cytokine gene expression by cultured cells will mimic expression induced *in vivo* during parasite infections, will be tested. Also tested will be the
hypothesis that glucocorticoids reduce gene-expression changes induced
during parasite co-culture and effect the growth of cultured cells.
4.2 Materials & Methods

For a more detailed explanation of methodology refer to chapter 6: materials and methods

4.2.1 Cell Culture

Rat small intestine cell lines IEC6, derived from the jejunum and IEC18 derived from the ileum were used in this study to be representative of the site of infection of H. bakerii in rats. IEC6 cells were grown cultured in high glucose (4500 mg/L) DMEM (Dulbecco’s Modified Eagle’s Medium – Invitrogen) supplemented with 5% FBS, 0.1 U/mL insulin, 50 µg/mL streptomycin and 50 U/mL penicillin. IEC18 cells were cultured in high glucose (4500 mg/L) DMEM supplemented with 5% FBS, 50 µg/mL streptomycin and 50 U/mL penicillin. Cell cultures were grown at 37°C and 8% CO₂ taking into account the altitude at which the laboratory is situated (1,000m above sea level).

4.2.2 DEX Dose Response

A dose response curve was generated using 10⁻⁴ M to 10⁻⁹ M dexamethasone (Sigma - DEX) in appropriate media for IEC6 and IEC18 cells described above. Cells were labelled with Cell Trace™ (life technologies) to measure cell proliferation. Cells were grown in 6-well cell culture dishes at 37°C +5%CO₂ for 4 days. Media was replaced after two days. Flow cytometer gating and voltages were set using mitomycin-c treated Cell Trace™ labelled cells. RNA was extracted from cell pellets snap-frozen in liquid nitrogen and stored at -80°C, using the RNeasy mini kit (Qiagen).

4.2.3 Response to Parasitic Gastrointestinal Nematodes

IEC6 cells were grown in 12-well cell culture inserts (Falcon) of 0.4 µM pore size containing Collagen I (Gibco) pads (2.3 mg/mL in high glucose DMEM), inserted into multiwall 12 well plate wells (Falcon). Cells were diluted to 4 x 10⁶ cells/mL in IEC6 media containing 0M, 1 x10⁻⁵M or 1 x10⁻⁶M DEX and 250µL of

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?
N.O. FENNER (2012)
this was applied to the collagen pads. Cells were allowed to attach overnight before the media was removed from insert. Worms were applied to the cells on collagen in IEC6 media containing 0M, 1 x10^{-5} M or 1 x10^{-8} M DEX. Worms were exsheathed and treated with antibiotics (see Chapter 6) before applying to cells. Approximately 50 exsheathed L3 larvae were added to each well insert.

4.2.4 Statistics
All cell culture experiments were designed so that each experimental group was in triplicate (n=3). The raw Ct values for each target gene were normalised to two reference genes using the Biorad GenEx freeware program. The resulting expression values were then imported to GraphPad Prism, to undertake statistical analysis and generate graphs. Two-tailed Student's t-tests were used to determine whether gene expression values differed significantly between experimental groups. For all tests significance levels were set at α=0.05. Significance is represented in figures with stars * = p<0.05, ** = p<0.001 and *** = p<0.001
4.3 Results

4.3.1 Effects of Glucocorticoids on Cell Growth
Glucocorticoids play an important role in accelerating the maturation of the gastrointestinal tract but also have pleiotropic effects on gastrointestinal crypt cells. Research has demonstrated that glucocorticoids may negatively effect the growth of gastrointestinal and other cell types. However, results obtained in a DEX response assay (Figure 4.2) contradict this, demonstrating that glucocorticoids at biological concentrations may have stimulatory effects on IEC6 cell proliferation. This is supported by data that suggests that chronic glucocorticoids have proliferative effects on jejunal crypt cells. These stimulatory effects were not seen however in the IEC18 cells (Figure 4.3). These results suggest IEC6 cells may be more responsive to glucocorticoids than IEC18 cells. Interestingly, examination of gene expression in these cells showed that both cell lines did change gene expression profiles in response to DEX (Figures 4.4 and 4.5). Expression of the GR gene was higher in IEC6 cells than in IEC18 cells suggesting a possible mechanism behind the growth response differences seen between the two cell lines. Interestingly, the presence of glucocorticoids did not downregulate all genes measured in IEC6 cells but selectively downregulated some pro-inflammatory cytokines (IL-1B, TNFa), whilst upregulating the TH2 cytokine IL33 and also the pro-inflammatory cytokine IL-6. Downregulation of IL-25 and GR was observed in both cell lines in response to DEX (Figures 4.4 and 4.5).

Cultured IEC6 cells showed a cell division/proliferation dose response to DEX (figure 4.2). In comparison to control, unsupplemented media, IEC6 cells grown in media with a DEX concentration of $1 \times 10^{-8}$M showed an increase in cell division with a division index of 2.6 ($p=0.03$) and a proliferation index of 3.6 ($p=0.04$). Similarly, at the higher DEX concentration of $1 \times 10^{-5}$M IEC6 cells had a division index of 9.3 ($p=0.01$) and a proliferation index of 9.5 ($p=0.05$) greater than cells grown in control medium. There were also between-concentration differences observed with the cell division/proliferation of IEC6 cells grown in
media containing DEX at $1 \times 10^{-5}$M being 6.7 ($p=0.03$) and 8.2 ($p=0.01$) times greater than cells grown in media supplemented with $1 \times 10^{-8}$M and $1 \times 10^{-9}$M DEX respectively. The proliferation indexes were not significantly different between cells grown at DEX concentrations $1 \times 10^{-5}$M, $1 \times 10^{-6}$M and $1 \times 10^{-7}$M respectively.

IEC6 cells were grown to confluence in media containing varying concentrations of DEX and gene expression of GR, IL-1β, IL-25, IL-33, IL-6 and TNFα were measured (figure 4.4). Overall data shows a dose dependant downregulation of GR, IL-1β, IL-25 and TNFα expression, whilst IL-33 and IL-6 may show a dose dependant up regulation due to DEX.

The expression of the GR was downregulated in a dose dependant manner when grown in media containing DEX. With respect to the control of media without the addition of DEX, there was a downregulation in GR expression. GR expression was reduced by 138% ($p=0.002$) in the $1 \times 10^{-4}$M group. The reduction in gene expression reduced as DEX concentration reduced where gene expression was decreased by 132% ($p=0.002$), 115% ($p=0.006$), 94% ($p=0.003$), and 67% ($p=0.04$) for concentrations $1 \times 10^{-5}$M, $1 \times 10^{-6}$M, $1 \times 10^{-7}$M and $1 \times 10^{-8}$M with respect to the control.

As with GR expression, IL-1β also showed downregulation of gene expression in a dose dependant manner. At a DEX concentration of $1 \times 10^{-4}$M GR expression was reduced by 138% ($p=0.01$) with respect to the control. Similar results were seen with DEX concentrations of $1 \times 10^{-5}$M and $1 \times 10^{-7}$M where expression was reduced by 244% ($p=0.003$) and 143% respectively ($p=0.002$).

TNFα was also significantly downregulated in all the DEX treatment groups. At a media DEX concentration of $1 \times 10^{-4}$M TNFα gene expression was downregulated by 160% ($p=0.002$), at $1 \times 10^{-5}$M downregulation was 132% ($p=0.006$), at $1 \times 10^{-6}$M downregulation was 109% ($p=0.008$), at $1 \times 10^{-7}$M downregulation was 144% ($p=0.002$) and at $1 \times 10^{-8}$M downregulation was 142% ($p=0.004$).

Dose dependant downregulation of IL-25 was observed with all treatment groups being significantly downregulated with respect to the control. At media
concentration of DEX at $1 \times 10^{-4}$M IL-25 expression was reduced by 116% ($p=0.03$) of the control. Similar results were observed with DEX concentrations $1 \times 10^{-5}$M through to $1 \times 10^{-8}$M with a 149% ($p=0.0001$), 142% ($p=0.0001$), 118% ($p=0.001$) and 100% ($p=0.0007$) reduction in expression.

In comparison to GR, IL-1β, TNFα and IL-25 gene expression response to DEX, the expression of IL-33 showed a positive relationship between gene expression and DEX concentration. At DEX concentration of $1 \times 10^{-5}$M IL-33 expression was upregulated by 19% ($p=0.04$) while at $1 \times 10^{-8}$M gene expression was upregulated by 22.5% ($p=0.01$).

As with IL-33, IL-6 also showed a positive relationship between gene expression and DEX concentration. At DEX concentrations of $1 \times 10^{-5}$M, $1 \times 10^{-6}$, and $1 \times 10^{-7}$M IL-6 gene expression was upregulated by 46%($p=0.01$), 47% ($p=0.01$), 43% ($p=0.02$) respectively.

IEC18 cells showed no change in cell division/proliferation in the presence of DEX in media (figure 4.3) Cell division/proliferation was constant throughout the DEX dose curve with concentration of DEX in media having no effect on cell division/proliferation.

IEC18 cells were grown to confluence in media containing varying concentrations of DEX and gene expression of GR, IL-1β, IL-25, IL-33, IL-6 and TNFα were measured figure 4.5). Overall data shows a dose dependant downregulation of GR, IL-25 and IL-6 expression.

Dose dependant downregulation of GR was observed at media DEX concentrations at $1 \times 10^{-4}$M, $1 \times 10^{-5}$M, $1 \times 10^{-6}$M and $1 \times 10^{-8}$M where GR expression was downregulated by 69% ($p=0.01$), 53% ($p=0.003$), 54% ($p=0.008$), and 29% ($p=0.04$) respectively.

Similar results were seen with IL-25 gene expression, where dose dependant downregulation of expression was observed. At media DEX concentrations of $1 \times 10^{-4}$M, $1 \times 10^{-6}$M, $1 \times 10^{-7}$M and $1 \times 10^{-8}$M IL-6 gene expression was downregulated by 44% ($p=0.01$), 31% ($p=0.02$), 55% ($p=0.009$) and 44% ($p=0.008$) respectively.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
With IL-6 gene expression in response to DEX treatment, only one treatment group differed significantly. At media DEX concentration of $1 \times 10^{-8}$ M IL-6 gene expression was downregulated by 102% ($p=0.04$).

There was no significant changes in gene expression of IL-1β and IL-33 observed at the various DEX treatment levels, whilst TNFα gene expression was below detectable limits (bdl).

**Figure 4.2: Effects of glucocorticoids on IEC6 cell basal growth.** Duodenal derived IEC6 showed a positive dose response to DEX concentrations. As a general observation IEC6 cell line proliferated at a faster rate when exposed to higher levels of DEX compared to low levels or DEX absence. Mean division and proliferation indices shown in response to increasing DEX concentration in media. Error bars indicate SEM. Significant difference to growth in unsupplemented medium noted by stars.
Figure 4.3: Effects of glucocorticoids on IEC18 cell basal growth. The ileum derived IEC18 cell line however showed no change in proliferation in response to DEX concentration. Mean division and proliferation indices shown in response to increasing DEX concentration in media. Error bars indicate SEM. Significant difference to growth in unsupplemented medium noted by stars.
Figure 4.4: Basal gene expression response of IEC6 cells to DEX. After growing IEC6 cells to confluence in media containing DEX, the gene expression of various cytokines was measured. Overall data shows a dose dependant downregulation of GR, IL-1β, IL-25 and TNFα expression, whilst IL-33 and IL-6 may show a dose dependant up regulation due to DEX. Mean relative gene expression of various genes shown in response to increasing DEX concentration in media. Error bars indicate SEM. Significant difference to expression in unsupplemented medium noted by stars.
Figure 4.5: Gene expression response of IEC18 cells to DEX. After growing IEC18 cells to confluence in media containing DEX, the gene expression of various cytokines was measured. Overall data shows a dose dependant downregulation of GR, IL-25 and IL-6 expression. There was no significant effect of DEX in IL-1β and IL-33 expression. TNFα expression was below detectable limits. Mean relative gene expression of various genes shown in response to increasing DEX concentration in media. Error bars indicate SEM. Significant difference to expression in unsupplemented medium noted by stars.
4.3.3 Gene expression changes in response to *H. bakerii* & the effects of Glucocorticoids

An examination of gene expression changes in cultured IEC6 cells occurring in response to parasite exposure was undertaken. By adding live, exsheathed *H. bakerii* larvae to the culture, both exposure to E/S products and physical disruption of cells were allowed to occur. In some of the culture wells, DEX was also added to the culture media to determine the effects of glucocorticoids on the response to parasite exposure. Results showed that in the absence of DEX, larvae induced cultured IEC6 cells to express pro-inflammatory cytokine (IL-1β, TNFa, IL-6) and IL-33 genes. The GR gene expression increased during the first day of exposure to parasites, but returned to basal levels for the remainder of the culture time examined. In contrast, the expression of IL-25 was unchanged in response to parasite exposure. The addition of DEX completely abolished the expression of GR, pro-inflammatory cytokines and IL-33 in response to parasites, but had no effect on IL-25 gene expression. The results are similar to *in vivo* observations (Chapter 3), showing that gastrointestinal cells in culture are capable of appropriate gene expression in response to parasite exposure. Also, glucocorticoids were shown to reverse this expression. This *in vitro* parasite infection model has therefore been shown to simulate the cytokine profiles found in tissues of whole animal experiments and in some circumstances may provide an alternative to animal trials. The high relative expression of IL-1β and IL-6 suggest that these cytokines are important in the initial cytokine signalling which is consistent with expression found in whole animal parasite challenges. IL-1β is a pro-inflammatory cytokine and important in the monitoring of the inflammation process by the HPA axis. IL-1β expression significantly increased during *H. bakerii* exposure in the (-)DEX group. With respect to day 0, IL-1β expression was 17 fold increased at day 1 (p= 0.000498), and 543 fold increased at day 4 (p= 0.010585).

The effects of glucocorticoids were simulated through addition of DEX to the...
The addition of DEX resulted in a 24 \( (p=0.001) \) and 677 \( (p=0.01) \) fold decrease in IL-1\( \beta \) expression respectively. IL-6 plays an important role in activating immune cells involved in the immune response. It has both anti-inflammatory and pro-inflammatory properties. IL-6 expression significantly increased during \textit{H. bakerii} in the (-) DEX group. With respect to day 0, IL-6 expression increased by 11 \( (p=0.006) \), 94 \( (p=0.004) \) and 114 \( (p=0.037) \) fold at days 1, 4 and 6 respectively.

The effects of glucocorticoids were simulated through addition of DEX to the growth media. At each timepoint IL-6 gene expression was 18 fold higher at day 0 \( (p=0.010) \), 220 fold higher at day 1\( (p=0.006) \), 1093 fold higher at day 4 \( (p=0.004) \) and 1318 fold higher at day 6 \( (p=0.036) \) in (-) DEX groups compared to (+) DEX.

TNF\( \alpha \) is a key pro-inflammatory cytokine involved in the activation of NFkB and AP-1. TNF\( \alpha \) expression in the (-) DEX group showed significant downregulation during the early stages of parasite exposure in culture, but this changed to upregulation after day 4. With respect to day 0, TNF\( \alpha \) expression decreased by 1.8 fold at day 1 \( (p=0.0009) \) and 2 \( (p=0.006) \) respectively, but increased by 13.5 fold at day 6 \( (p=0.0004) \).

The effects of DEX resulted in a decrease in TNF\( \alpha \) expression by 1.3 fold at day 1 \( (p=0.017) \) but increased 6.1\( (p=0.043) \) and 18 \( (p=0.000018) \) fold at day 4 and 6 respectively.

The effects of glucocorticoids are mediated through the activity of GRs. The activated GR dimer acts as a transcription factor important in many genes involved in metabolism and immune and stress responses.

GR expression varied slightly during parasite co-culture. GR expression was 2 \( (p=0.00002) \) fold higher at day 1 but 1.1 \( (p=0.04) \) fold lower at day 2.

The effects of DEX on GR gene expression only differed significantly at day 1, with (-)DEX GR expression being 2.6 fold higher than (+)DEX \( (p=0.003) \).

IL-33 expression in the (-) DEX group showed significant changes during co-culture. With respect to day 0, IL-33 expression decreased 1.4 fold at day 1 \( (p=0.005) \) but by day 2 had increased 2.2 \( (p=0.024) \) fold, followed by a 30
(p=0.017) fold increase at day 4. At day 6 there was no significant difference in IL-33 expression.

The addition of DEX to the cell growth media affected IL-33 expression significantly. IL-33 expression was 18 (p=0.0004), 16 (p=0.0001) 17 (p=0.007), 586 (p=0.016) fold higher in the (-)DEX group, with respect to the (+)DEX group at days 0 through to 4, with no significant difference seen at day 6.

![IL-1b expression ratio](image)

Figure 4.8: IL-1β gene expression of IEC6 cells in response to *H. bakerii* infection. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX. IL-1β gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between (-)DEX and (+)DEX co-cultures are represented by stars. Standard error of the mean is represented by error bars.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

191
Figure 4.9: IL-6 gene expression of IEC6 cells in response to *H. bakerii* infection. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX. IL-6 gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between (-)DEX and (+)DEX co-cultures are represented by stars. Standard error of the mean is represented by error bars.
Figure 4.10: TNFα gene expression of IEC6 cells in response to *H. bakerii* infection. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX. TNFα gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between (-)DEX and (+)DEX co-cultures are represented by stars. Standard error of the mean is represented by error bars.
Figure 4.11: GR gene expression of IEC6 cells in response to *H. bakerii* infection. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX. GR gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between (-)DEX and (+)DEX co-cultures are represented by stars. Standard error of the mean is represented by error bars.
Figure 4.12: IL-25 gene expression of IEC6 cells in response to H. bakerii infection. IEC6 cells were grown to 80% confluence with a co-culture of H. bakerii larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX. IL-25 gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between (-)DEX and (+)DEX co-cultures are represented by stars. Standard error of the mean is represented by error bars.
Figure 4.13: IL-33 gene expression of IEC6 cells in response to *H. bakerii* infection. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX. IL-33 gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between (-)DEX and (+)DEX co-cultures are represented by stars. Standard error of the mean is represented by error bars.
4.4 Discussion

The experiments described in this chapter demonstrated that glucocorticoids affect gastrointestinal epithelial cell growth, basal cytokine expression and cytokine gene expression responses to parasite co-culture. Furthermore, results demonstrated that glucocorticoids effects gene expression of some but not all cytokines in the cell culture system, and that different cell lines can vary in their response to glucocorticoids.

4.4.1 Effects of glucocorticoids on cell growth & basal gene expression

By measuring the cell proliferation of the cell lines IEC6 and IEC18 grown in the presence of DEX it was evident that cell lines differ in their growth response to glucocorticoids and that glucocorticoid concentration has an effect on cell growth for IEC6 only. IEC6 cells showed a positive relationship between glucocorticoid concentration and cell growth. At concentrations of $1 \times 10^{-8}$M and $1 \times 10^{-5}$M IEC6 cells proliferated at a greater rate than the control, but IEC18 cells did not show this response. This suggests that cells respond to glucocorticoids differently depending on tissue origin, because IEC6 cells are derived from duodenal epithelial crypt cells [24] and IEC18 cells are derived from ileum epithelial crypt cells [36]. Examining IEC6 cells in isolation, it was evident that glucocorticoids promoted proliferation in a dose dependant manner. In fact, addition of DEX at $1 \times 10^{-5}$M to growth media tripled cell proliferation. Therefore it may be that glucocorticoids promote cell division in the jejunum, but not the ileum. These results were consistant with Gunin & Nikolaev [39], where glucocorticoids promoted cell division in jejunal cells in chronic glucocorticoid treatments and where adrenalectomy resulted in impaired growth of small intestine epithelium in vivo [37]. The effects of glucocorticoids on the gene expression of c-jun and c-fos, a crucial component in the promotion of cell growth through the AP-1 transcription factor pathway, demonstrated that glucocorticoids induced c-jun expression whilst c-fos was unaffected by the presence of glucocorticoids [26]. It is hypothesised that it was through this pathway that glucocorticoids were taking effect in IEC6 cells studied in this

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

197
chapter where glucocorticoids promoted cell proliferation. Further experimentation would however be required to examine this. There may be other differences between these cell lines that explain the observations and more work would be justified to explore this observation further. Of further interest, these findings are conflict with other research where hydrocortisone inhibited growth of cells in culture [36].

As well as examining the effect of Dex on cell growth, the effect on gene expression was also examined. Overall, both cell lines showed significant changes in gene expression for selected genes when DEX was added to growth media. Both cell lines showed downregulation of GR and IL-25, whilst downregulation of IL-1β and TNFα was observed in IEC6 cells only. Interestingly, in IEC6 cells only the expression of IL-33 and IL-6 was upregulated in response to glucocorticoids, showing that glucocorticoids have both stimulatory and inhibitory effects on gene expression.

It is consistent with the hypothesis that glucocorticoid responses are involved in TH2 response implementation and subsequent TH1 inhibition [40, 41], results demonstrated that in IEC6 cells genes associated with the TH2 response, IL-6 and IL-33, were upregulated in the presence of glucocorticoids whilst TH1 response associated genes, IL-1β and TNFα, were downregulated in the presence of glucocorticoids. Studies have demonstrated that IL-12 is inhibited by glucocorticoids at the APC level therefore reducing IFNγ synthesis and TH1 expansion [42, 43]. However, the synthesis of IL-10, a major TH2 cytokine, was demonstrated to be unaffected by glucocorticoids at a monocyte level and upregulated at a lymphocyte level [43, 44]. This demonstrated the differing effects of glucocorticoids on the TH1/TH2 balance. This study described in this chapter also demonstrated similar findings where IL-6 and IL-33 were upregulated in IEC6 cells in the presence of DEX, whilst IL-1β and TNFα was downregulated under the same conditions.

Both cell lines demonstrated downregulation of the GR gene in a DEX dose-dependent manner. This is likely to be a result of a negative feedback
mechanism regulating glucocorticoids and the glucocorticoid receptor. This phenomenon, known as GR autoregulation, was reviewed by Svec et al. [45] describing glucocorticoid induced downregulation of GR in both cell lines and in tissues. GR regulation is complex and has many levels of regulation both at a transcriptional and functional level. It is unclear how the dynamics of the GR and the AP-1 cell growth pathway interacted and presents a further area for investigation. Also of significance is the findings that glucocorticoids may also be synthesised at a gastrointestinal level, with gastrointestinal crypt cells being a source of extra-adrenal glucocorticoids [46, 47]. This chapter has shown GR autoregulation in IEC6 and IEC18 cell lines.

4.4.2 The co-culture of IEC6 cells and H. bakerii larvae
The co-culture of IEC6 cells and H. bakerii larvae aimed to replicate a parasitic infection of the rat in vitro. The addition of DEX to the growth media aimed to determine the effects glucocorticoids had on the in vitro system, and determine possible roles for glucocorticoids in the immune response to nematode parasites. Intestinal epithelial cells are an important target cell due to their location at the site of infection and as a first line of defence in the initial immune response. Intestinal epithelial cells are important primary immune cells being receptive to cytokines and being able to express chemokines and receptors [4, 5].

Of the genes examined in the in vitro system IL-1β, IL-6, TNFα, and IL-33 showed an upregulation in expression over time during co-culture, whilst GR expression increased significantly at day 1 but not for the remaining timepoints. The expression of IL-25 was unchanged during co-culture. The addition of DEX to the growth media significantly reduced gene expression in response to nematode larvae, where the upregulation of IL-1β, IL-6, TNFα and IL-33 gene expression was repressed by the presence of glucocorticoids. However, the addition of glucocorticoids to media had no effect on IL-25 expression and limited effect on GR expression.
This chapter aimed to replicate a parasitic infection *in vitro*, and although not conclusive, the results are encouraging, with some alignment between *in vivo* and *in vitro* observations. To an extent, results from this chapter and chapter 3 were similar despite the differences in the systems examined. Overall, the two chapters demonstrated the important role of IL-6 and IL-33, whilst suggesting that IL-25 may not be involved in the epithelial response to parasitic nematode infection. Further work would be needed to assess the viability of the system as an *in vitro* model of parasitic infection. For example, in this study the apoptotic activity of cells exposed to gastrointestinal nematode larvae was not measured, however it is possible that nematode secreted products might increase the rate of apoptosis based on the findings of Kuroda et al [48].

This chapter also aimed to analyse the effects of glucocorticoids on cell growth and gene expression of gastrointestinal cell lines *in vitro*, using DEX as a model glucocorticoid. From the results it is possible that glucocorticoids have differing effects on cells dependant on the tissue of origin. This was evident in the stimulation of cell proliferation by DEX in IEC6 cells and not in IEC18 cells. Also of interest were observations of changed gene expression in response to DEX. The overall thesis hypothesis stated that glucocorticoids play a role in 1.) downregulating the TH1 immune response and 2.) stimulating the TH2 immune response. The results of this chapter present evidence suggesting that this hypothesis holds some validity. Of IEC6 cells grown in the presence of glucocorticoids it was evident from gene expression analysis that TH1 associated genes were downregulated while favouring TH2 gene expression. This suggests that glucocorticoids favour the generation of the TH2 immune response without cross stimulating the TH1 immune response. To assess these findings further it would be interesting to increase the selection of genes analysed to include important TH2 genes such as IL-10 and IL-4, and TH1 genes such as IFNγ and IL-2. Also a timecourse over a number of days would assist in demonstrating how the response was generated. Additionally, the use of a GR blocking molecule would act as an additional negative control to determine whether the pathway is through the GR or through other pathways.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
4.5 Appendix

4.5.1 Gene expression changes in response to *H. bakerii* co-culture

The following figures represent the gene changes that occurred during a *H. bakerii* co-culture over 6 days cultured in media not containing DEX.

![IL-1β gene expression](image)

**Figure A4.1: IL-1β gene expression in response to *H. bakerii* co-culture without DEX in media.** IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX which is show in this figure. IL-1β gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between timepoint 0 and other timepoints are represented by stars. Standard error of the mean is represented by error bars.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure A4.2: IL-6 gene expression in response to *H. bakerii* co-culture without DEX in media. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX which is shown in this figure. IL-6 gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between timepoint 0 and other timepoints are represented by stars. Standard error of the mean is represented by error bars.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure A4.3: TNFα gene expression in response to *H. bakerii* co-culture without DEX in media. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX which is show in this figure. TNFα gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between timepoint 0 and other timepoints are represented by stars. Standard error of the mean is represented by error bars.
Figure A4.4: GR gene expression in response to *H. bakerii* co-culture without DEX in media. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX which is show in this figure. GR gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between timepoint 0 and other timepoints are represented by stars. Standard error of the mean is represented by error bars.
Figure A4.5: IL-25 gene expression in response to *H. bakerii* co-culture without DEX in media. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX which is shown in this figure. IL-25 gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between timepoint 0 and other timepoints are represented by stars. Standard error of the mean is represented by error bars.
Figure A4.6: IL-33 gene expression in response to *H. bakerii* co-culture without DEX in media. IEC6 cells were grown to 80% confluence with a co-culture of *H. bakerii* larvae on collagen pads with media containing DEX. The control consisted of cells grown with parasites in media absent of DEX which is shown in this figure. IL-33 gene expression was measured and reported as an expression ratio of target to reference gene. Measurements were taken at 5 different timepoints at days 0, 1, 2, 4, and 6. Significant differences between timepoint 0 and other timepoints are represented by stars. Standard error of the mean is represented by error bars.
4.5.2 Cell line morphology

Changes in cell morphology in response to glucocorticoids have been documented. These changes include reduction in cell thickness, increased surface area and volume, reduction of intracellular spaces and reduced thickness and elongation of microvilli. Here both IEC6 and IEC18 cells were H&E stained and examined under light microscope. Cells were grown to approximately 80% confluence on glass slides. Both IEC6 and IEC18 cell lines have similar appearance with the cell nucleus taking on an oval appearance, whilst the cytoplasm is unregular in shape at 80% confluence. Cell dimensions are approximately 20µM by 28µM with the nucleus taking up approximately half of the cell with a dimension of 10µM by 14µM.

Figure A4.7: IEC6 morphology. Morphology of IEC6 cells grown to 80% confluence on microscopy slide. (a) 2.5x (b) 10x (c) 20x and (d) 40x
Figure A4.8: IEC18 morphology. Morphology of IEC18 cells grown to 80% confluence on microscopy slides. (a) 2.5x (b) 10x (c) 20x and (d) 40x
4.6 References


17. Schmitz, J., et al., IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. Immunity, 2005. 23(5): p. 479-490.


Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
46. Mueller, M., et al., The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes? 

N.O. FENNER (2012)

CHAPTER 5: Discussion & Conclusion

5.1 Experimental Overview

Alternatives to modern anthelmintic drugs will be required in the future as anthelmintic drug resistance develops in both human and veterinary health [1, 2]. An understanding of the role of the HPA axis in regulating an immune response to parasitic infection may provide an avenue for improved anti-parasitic strategies, and provide novel phenotypes for selective breeding. This thesis aimed to examine the interactions between the HPA axis and the immune system in the context of a gastrointestinal parasitic infection. It was hypothesised that the activity of the HPA axis would play a significant role in shaping and regulating the immune system through the activity of glucocorticoids. Both in vivo and in vitro experiments were designed to assess the immune outcomes of differing glucocorticoid concentrations on resistance to parasite invasion. Chapters 2 and 3 explored the differences in immune responses between Lewis and Fischer rat strains to novel antigens and H. bakerii respectively, whilst chapter 4 explored the response to H. bakerii at a cellular level manipulated through corticosteroid treatments. All three experiments were designed to assess the role of the HPA axis in shaping and regulating the immune response to parasitic infection, more specifically the effects of corticosterone on the shape of the immune response generated. The literature suggests that glucocorticoids may help to determine the type of immune response generated through positively and negativity affecting the cytokine balance [3-10].

In Chapter 2 the role of the HPA axis in regulating the immune response to ovalbumin, where differences in responses of Fischer and Lewis rats were examined. The immune responses to ovalbumin were modified through the use of adjuvants designed to change the nature of the cytokine profiles generated. It
was hypothesised that the immune response to specific model antigens will differ between the rat strains due to their inherent HPA axis profile. Ovalbumin was injected subcutaneously with either Aluminium hydroxide (AO) or Freund's complete/incomplete adjuvant (FO) at days 1, 28 and 33 of a 38 day immunisation regime. Plasma was collected at various times and immunological and neuroendocrine tissues were collected at the termination of the experiment. Results showed that whilst corticosterone levels differed between strains, there were no effects of antigen injection on plasma corticosterone. Changes were observed in gene expression in HPA axis tissues where hypothalamus CRH was upregulated in the Fischer strain in response to AO and similarly, adrenal gland expression of MC2R was upregulated also in the Fischer strain in response to AO. The Lewis strain showed no changes in gene expression in HPA axis tissues. This showed that the Fischer strain may be more responsive to immunological stress however this change in gene expression did not translate into corticosterone output changes. As well as changes in HPA axis tissue, there were also changes in cytokine expression in the PLN, where IFNγ was upregulated in the Lewis strain in response to both AO and FO, while the Fischer strain was unresponsive. On the other hand IL-10 was upregulated in the Fischer strain in response to AO, whilst the Lewis strain was unresponsive. These results suggested that the TH1 response at the PLN was stimulated in the Lewis strain whilst the TH2 response was stimulated in the Fischer strain. This result is consistent with the TH2 promoting effects of neuroendocrine activity [4-8], demonstrating that the corticosterone levels of the Fischer strain in this instance promoted the establishment of the TH2 response. In further detail, the Fischer results suggest that the shape of the immune response is being influenced by glucocorticoid levels where the higher basal glucocorticoid levels of the Fischer corresponded to the significant upregulation of HPA axis genes as well as upregulation of IL-10 and the lack gene expression changes in pro-inflammatory TH1 cytokines such as IFNγ. Although results were limited there was some evidence of the HPA axis influence on shaping the immune response. It may be the basal levels of HPA axis activity, rather than HPA axis...
responsiveness that was important in influencing the cytokine profiles demonstrated.

To further understand the role of the HPA axis in response to antigens it was suggested that alternatives to ovalbumin be used that induced a greater degree of pathology. It was evident that the antigen injection regime did not provide a significant immune stimulation as it did not cause any significant physiological response such as increased corticosterone levels or body temperature. Similarly, modulation of the system through the use of a slow release antigen might also allow a more thorough investigation. The use of pharmacological levels of synthetic corticosterone (DEX) to modulate the glucocorticoids levels would be considered to be inappropriate due to the high degree of activity of DEX in biological systems. It is hypothesised that DEX would cause immunosuppressive outcomes which are to be avoided, since it is anti-inflammatory yet pro-immunological responses that are desired to result in an anti-TH1 but pro-TH2 response. To overcome this is it is suggested that a slow release CRH or ACTH mechanism be employed to achieve sustained and elevated corticosterone levels.

Where chapter 2 utilised simple antigens to initiate an immune response, chapter 3 utilised live parasitic gastrointestinal nematode larvae to initiate a TH2 immune response. *H. bakerii* is a laboratory strain of the natural mouse parasite *H. polygyrus* [11]. While *H. polygyrus* naturally infects mice, the parasite can also initiate infections in rats although a strong immune response characterised through inflammation and connective tissue genesis normally prevents the nematodes reaching the adult stage. Chapter 3 aimed to examine how the HPA axis influences the way a TH2 immune response is generated and regulated. As in chapter 2, Fischer and Lewis rats were utilised due to their differing levels of HPA axis activity. Infections in these rats strains did not become patent as no nematode eggs were observed in faeces throughout the post infection period, however parasite DNA was present in the infected animals intestinal tissue post mortem, implying the presence of arrested larvae as have been observed.
previously [12]. Parasite infection had no effect on plasma corticosterone levels in either rat strain, though there were significant differences in blood corticosteroid concentrations between the strains, irrespective of infection status. Body temperature was also monitored and found to be unaffected by parasite infection. Examination of gene expression levels in tissues showed that there were significant differences in gene expression between uninfected Fischer and Lewis and also in the response to infection in the two rat strains. In the small intestine IL-33 was upregulated by infection in both strains whilst IL-25 was upregulated by the Fischer strain and downregulated by the Lewis strain. This suggests that IL-33 is involved at the site of infection where early expression may be crucial in the release of IL-6 from cells such as mast cells and eosinophils [13]. IL-33 expression may be conserved between the two strains as IL-33 is essential in stimulating the TH2 immune response [14, 15]. However, IL-25 has also been shown to stimulate the TH2 immune response [16], so the differences in expression between the two strains may present a significant difference in immune responses between strains, possibly regulated by basal HPA axis activity, however this difference did not manifest as a difference in parasite susceptibility. Other differentially expressed genes included IFNγ and STAT-4 in the spleen, both of which were downregulated in the Lewis strain. STAT-4 is an important transcription factor for the TH1 expression and is involved in IFNγ gene expression. It is therefore logical to observe downregulation of IFNγ concurrently with STAT-4 downregulation. STAT-4 downregulation was also observed in the MLN by both strains. This therefore demonstrates that the Lewis strain is actively suppressing the TH1 response at the level of the spleen and MLN whilst the Fischer strain is only suppressing TH1 at the MLN, suggesting that regulation of the T helper response may be at the level of the spleen in Lewis.

Observing the basal levels of cytokine gene expression in the small intestines shows distinctly higher basal levels of pro-inflammatory cytokine genes in the Fischer strain. The Fischer strain had higher basal levels of both IL-1β and
TNFα. However, in both strains the expression of pro-inflammatory cytokines linked with the TH1 response (IL-1β and TNFα) were unchanged in response to parasite infection. Whilst IL-1 and TNFα were unchanged in both strains IL-6 was upregulated in both strains. This firstly demonstrates that both strains generate an inflammatory response lead by IL-6, without TH1 pro-inflammatory cytokines. However, the Fischer strain was shown to have higher basal levels of TH1 pro-inflammatory cytokines suggesting that the Fischer strain may be more susceptible to TH1 inflammation.

There were also examples of upregulation of genes involved in the immune response against parasite infection that were conserved between the two strains. One example of this was the expression of the TH2-favouring IL-6 in the small intestine, the MLN and the spleen. Whilst upregulation of IL-6 was seen in the small intestine and the MLN in both strains, IL-6 was downregulated in the spleen. Although no cell tracking or FACS was conducted in this chapter it is suggested that such an experiment be conducted to ascertain the origins of IL-6 expression. It is possible that the change in IL-6 expression may be due to migrating macrophages and lymphocytes. These cells may be migrating from the spleen to the MLN and the small intestine leaving the spleen depleted of IL-6 expressing cells causing IL-6 downregulation in the spleen but upregualtion in the small intestine and the MLN (figure 5.1). This may demonstrate that the lymphatic response is important in the primary response to parasites and that the spleen may be a major source for IL-6 producing effector cells with the small intestine and the MLN being the destination for these cells. Similar results have been observed where *H. polygyrus* infection increases the B cell numbers in the MLN [17]. Other TH2 cytokines were also expressed in response to parasite infection in both strains. IL-13 was shown to be upregulated in both the MLN and the spleen in both strains while IL-10 was upregulated in both strains in the MLN. *H. polygyrus* infection has been shown to increase IL-13 in mucosal tissue, without IL-4 or IL-5 [18, 19]. This showed that although each strain had differing levels of basal HPA axis activity, this did not affect the core anti-parasite immune response, with IL-6, IL-10 and IL-13 expression being
conserved and upregulated in both strains [18-20].

Chapter 3 aimed to examine immune and neuroendocrine responses of Fischer and Lewis strain rats to the gastrointestinal nematode *H. bakerii* and it was hypothesised that the differences in HPA axis activity would result in differing immune response profiles in terms of HPA axis output, body temperature and gene expression of neuronedrocrine and immunological genes. Although there was evidence supporting the hypothesis there was also evidence against it. The main result which suggested the hypothesis should be rejected was the lack of a systemic change in HPA axis activity, such as increased plasma corticosterone concentration in response to parasite infection. Rather, the results suggested that the differences in the basal level of HPA axis activity could translate into subtle differences in the type of immune response observed. Neither strain demonstrated parasite susceptibility with both strains able to clear the parasite infection. However, in analysing the gene expression results it was evident that although the Fischer strain rats mounted an adequate immune response against *H. bakerii*, it might be less robust than that mounted by the Lewis strain animals.

To further this research it is suggested that the larval dose be increased, as a greater dose might result in increased pathology which in turn may help to stimulate the HPA axis. Secondly, the experiment only examined the primary response to *H. bakerii* so it is suggested that the experiment be repeated using a number of distinct infections in each experimental animal to simulate a secondary/tertiary response. Thirdly, this chapter was limited in the fact that only gene expression was measured and these results were not able to be validated through protein quantification techniques. Therefore, it is also suggested that qPCR results be validated through protein quantification to determine whether regulation occurred post transcription.
Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Figure 5.1: Gene expression changes in response to parasite infection. IL-6 was upregulated in the small intestine and MLN, but downregulated in the spleen. This is hypothesised to be due to the migration of IL-6 expressing cells from the spleen to the small intestine and MLN, leaving the number of IL-6 expressing cells in the spleen depleted. Confirmation of this theory would require \textit{in vivo} cell tracking techniques.

\textit{Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?}

\textit{N.O. FENNER (2012)}
The aim of chapter 4 was to examine in more detail the response of epithelial cells from the small intestine to *H. bakerii*. The experiment was carried out in vitro using a published methodology [21], however with rat cell lines, *H. bakerii* larvae and the use of DEX to examine the effect of glucocorticoids. The chapter was divided into two discrete but linked experiments. Firstly, both IEC6 and IEC18 cells were grown on collagen pads with differing levels of DEX included in the growth medium. Results from this experiment demonstrated that the two cell lines responded differently to the presence of DEX. Overall, both cell lines responded by downregulating pro-inflammatory cytokines and the GR. However, in the IEC6 cell lines cytokines involved in the TH2 response, IL-6 and IL-33 were actually upregulated in response to DEX. Therefore, this demonstrated that cell lines have differing responses to glucocorticoids and that in IEC6 cells the presence of glucocorticoids stimulate the TH2 response in terms of upregulation of IL-6 and IL-33.

Secondly, IEC6 cells were utilised in an experiment where cells were grown on collagen pads, either in the absence or presence of DEX, and exsheathed L3 larval stage *H. bakerii* added directly to the culture. The addition of parasites to the culture resulted in the upregulation of pro-inflammatory cytokines and IL-33. However, the addition of DEX to the media removed the gene expression response to larval co-culture. Interestingly, neither IL-25 nor GR expression was affected by larval co-culture and this effect was unchanged with the addition of DEX. These results demonstrated that co-culturing IEC6 cells with *H. bakerii* was possible and that the system could mimic a pro-inflammatory response possibly stimulated by IL-33.

Therefore, the chapter 4 results suggest that it is possible to replicate important aspects of the in vivo site of infection-parasite interface in an in vitro cell culture system. However, the two cell lines had divergent responses to DEX and therefore the use of multiple cell lines must be considered in future work. Interestingly, in both chapter 3 and chapter 4 there was a distinct role for IL-33 in generating a pro-inflammatory response. It is known that IL-33 is one of the key initial cytokines expressed by damaged cells and is instrumental in initiating...
the inflammatory response. As in chapter 3, the experiments in chapter 4 demonstrated that IL-6, IL-1β and TNFα were expressed concurrently with IL-33 expression, however critical for this study was the fact that when TH1 linked IL-1β and TNFα expression was downregulated in response to glucocorticoids, IL-33 and IL-6 were actually upregulated. This presents evidence supporting the role of the HPA axis in shaping the immune response favouring the TH2 response.

**Figure 5.2: The concentration of glucocorticoids shape the immune response.** The concentration of glucocorticoids shape the immune response through selectively downregulating TH1 pro-inflammatory cytokines (IL-1β and TNFα) while upregulating TH2 cytokines (IL-6). Gastrointestinal nematode parasites damage intestinal epithelial cells which response via IL-33 expression. IL-33 initiates the pro-inflammatory response triggering pro-inflammatory cytokine expression in intestinal epithelial cells, eosinophils, basophils and mast cells. The shape of the immune response depends on the glucocorticoid concentration with low glucocorticoid concentrations favouring IL-1β, IL-6 and TNFα expression whilst high glucocorticoid concentrations favouring IL-6 expression only.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
5.2 Conclusions

The results obtained from these experiments suggest the HPA axis may play a role in shaping and regulating the immune response to parasites. In contrast, neither model antigens nor live parasites stimulated increases in plasma glucocorticoids. This therefore suggests that basal HPA axis activity might be more important in regulating the differences in gene expression observed in response to *H. bakerii* in the Lewis and Fischer rat strains.

These results may have practical implications for the selective breeding of livestock to maximise parasite infection resistance. It might be desirable to alter HPA axis function through selection to achieve lines of animals with characteristics similar to the Fischer rat strain. However, whilst resistance to parasitic infection might be increased what would be the outcomes for resistance to bacterial and viral infections? Similarly, would production traits such as body mass and temperament be compromised? Similarly, breeding programs focussed on resistance alone, might unknowingly alter HPA axis characteristics, and this might also be an undesirable outcome. It seems evident therefore that selecting for parasite resistance based on the the activity of the neuroendocrine system would be illogical before properly evaluating the potential costs incurred by altering other health and production traits. Therefore, a pharmacological approach might be more appropriate at least in the short term, whereby subtle HPA axis modulation might allow for favourable modulation of responses to parasites without incurring a production cost. This approach might also be useful for medical applications.

The ability of an individual to cope with a stressful immunological event relies on the processes set in motion from the detection of the stressful event, initialising the immune response, regulating the immune response through glucocorticoid synthesis and regulating glucocorticoid synthesis through negative feedback. Detection of increased stress levels, such as reception of pro-inflammatory
cytokines and neuronal signals at the hypothalamus and hippocampus, initiates an increase in HPA axis activity resulting in the production of glucocorticoids. Glucocorticoids have a wide range of effects including inhibiting the synthesis of certain cytokines, such as IL-12 and IFNγ, whilst allowing other cytokines such as IL-10 to operate unabated. This results in a shift from TH1 mediated inflammation to TH2 mediated antibody production [22, 23]. Detection of immunological stress occurs in the hypothalamus and also may involve the hippocampus. Detection of IL-1β receptors and GR in these tissues suggests a direct relationship between systemic pro-inflammatory cytokines and HPA axis activity [22, 24] [25]. Similarly, the HPA axis negative feedback mechanism, in place as a mechanism to prevent over supply of glucocorticoids through self-regulation, may involve both the hypothalamus and hippocampus [24, 26-29]. This project also found evidence of a role for the hypothalamus in responses to parasite infection. The upregulation of both IL-1β and IL-1R1 in the hypothalamus in response to parasite infection suggests that the IL-1 pathway is involved in regulating the neuroendocrine response. Similarly, upregulation of IL-1β in the hippocampus also suggests that the hippocampus response may be complementing the response of the hypothalamus. It has been suggested that the hippocampus may act as a detector of stress and a site for important negative feedback, relaying information into the HPA axis via the hypothalamus [27, 29, 30]. The results obtained in chapter 3 support this, suggesting that IL-1β may act as a signalling molecule in the brain and that the presence of GR and IL-1R1 in the hippocampus suggests a role for the hippocampus in HPA axis regulation.

The hippocampus and the hypothalamus are detecting immunological stress via circulatory pro-inflammatory cytokines and possibly activated neuronal activity, responding with increased HPA axis and resulting in glucocorticoid synthesis. However, in both chapter 2 and chapter 3 immunological stimulation provided by a model antigen or complex parasites, failed to induce an increase in glucocorticoid synthesis. Therefore, changes in the immune response in terms of cytokine profiles occurred independent of glucocorticoid concentration. This
was further investigated with *in vitro* examination of intestinal epithelial cells in co-culture with *H. bakerii*, where high levels of glucocorticoid could block gene expression responses. The three experiments together suggest that glucocorticoids at low levels similar to those seen in Fischer rat plasma can influence the immune response favouring the TH2 immune profile (increasing IL-6, IL-10, IL-13 and IL-33 gene expression and reducing IL-1β and TNFα expression), whilst lower (Lewis rat Chapter 3) or higher (cell culture work, Behnky and Parish, 1979 [12]; Cross 1960 [31]) glucocorticoid concentrations stimulate less or suppress TH2 responses respectively.

The TH1 mediated immune response to bacterial and viral antigens is well characterised and documented [32-34]. It classically involves antigen presentation via MHC class II by APC to naïve CD4+ T lymphocytes. This promotes TH1 differentiation and the production of type 1 cytokines. The TH2 mediated immune response differs from this classical APC-T cell hypothesis. Dendritic cells are known to be poor producers of IL-4, the primary cytokine in the differentiation of naïve CD4+ cells to TH2 cells and the key driver of the TH2 response. Where it was previously thought that a number of accessory cells were involved including eosinophils, basophils, and CD8+ cytotoxic T cells, it is now evident that basophils are a central cell type in antigen presentation in TH2 differentiation [35-37]. Furthermore, basophils are important sources of IL-4 and IL-6, important in the differentiation of CD4+ and CD8+ T cells [37]. Basophil expression of IL-6 in the spleen had been previously demonstrated in the mouse in response to *S. venezuelensis* [37]. Results demonstrated a decrease in the expression of IL-6 in the spleen, which may in part be explained by a migration of IL-6 expressing basophils away from the spleen. Also the origin of IL-13 in parasite infection may have also been from activated basophils through the Ag/IgE complex activation. Activation of the Ag/IgE complex also suggests a role for mast cells and eosinophils in cytokine production and effector responses [37]. It was evident that *H. bakerii* infection induced a TH2 response in both rat strains despite differences in basal HPA axis activity. Further experimentation suggested that IL-33 and IL-25 may be involved in generating
or amplifying this TH2 response. Both IL-33 and IL-25 are known to be involved in generation of the TH2 response [15, 16, 38]. Interestingly, glucocorticoids have a direct role in regulating IL-25 expression through the NF-κB pathway [39]. IL-33 was found to be expressed at the site of infection in both strains despite HPA axis basal activity. This was further investigated through IEC6 cell co-stimulation with live *H. bakerii*. This also generated an IL-33 response through increased expression in these cells. This response was nullified by glucocorticoid treatment, however in the absence of *H. bakerii*, the gene expression of IL-33 in IEC6 cells was unaffected by glucocorticoids.

In contrast to IL-33, expression of IL-25 was differentially expressed between the two rat strains Fischer and Lewis. Parasite infection in Fischer rats resulted in downregulation of IL-25, whilst expression in the Lewis strain was upregulated. This suggested that glucocorticoids may be involved in regulating IL-25 expression. However, *in vitro* experimentation showed that IL-25 expression was unaffected by co-stimulation by *H. bakerii* in the presence or absence of glucocorticoids. This therefore suggested that IL-33 may be more involved in generating the immune response to parasites than IL-25. In addition IL-33 was more susceptible to glucocorticoid mediated downregulation than IL-25. The susceptibility of IL-33 to glucocorticoid downregulation may be due to its role in localised inflammation [40, 41]. Post mortem, *H. bakerii* infected rats had intestines which appeared a darker pink colour and were thicker in comparison to uninfected rats. This localised inflammation of the gastrointestinal tract, in response to parasite infection, probably is responsible for inducing IL-33 expression [42, 43]. Although not evident visually, the differing glucocorticoid concentrations in the two rat strains may have subtly altered the inflammation resulting in the divergent IL-25 expression observed.

The expression of these cytokines may have subsequently stimulated the immune response through cells such as eosinophils, mast cells and importantly basophils [13, 44-46]. The inflammation signal may be amplified through the IL-33 and IL-25 pathways, leading to the generation of the TH2 response through basophil antigen presentation and Ag/IgE interaction of eosinophils and mast
cells. Rats from both strains were able to mount an effective immune response including IL-6 and IL-13 gene expression, even though the IL-25 responses in the intestine differed. This suggests that IL-25 was less important than IL-33 in the response to *H. bakerii*. Monitoring of this early inflammation response by the HPA axis, more precisely the [47] hippocampus and hypothalamus monitoring of pro-inflammatory cytokines, results in increased HPA axis activity resulting in the downregulation of the early innate inflammatory response, favouring a TH2 response with antibody production.

Of interest to the author, however not perused in experimentation in this thesis, is the implication of prenatal exposure to stressors and how this would impact upon immune function. It is known that certain stressors during the prenatal period can result in altered immune and HPA axis function [47-54]. Essentially, exposure of the mother during certain periods of pregnancy may result in a resetting of the HPA axis in the offspring resulting in lowered HPA axis responsiveness [47, 55]. In the context of this thesis, prenatal exposure of pregnant rats with higher basal corticosterone levels to stressors may result in the offspring having a phenotype which consists of lower HPA axis responsiveness of that of its parents. Therefore, this suggests that prenatal stressors may result in a reduction of the ability of the offspring to generate a robust TH2 response, as seen with comparisons between Fischer and Lewis rat strains. In practical terms this may reduce the resistance of a strain/breed to parasite infection. Similarly, strains which are considered to have heightened HPA axis responses might be reduced to an 'optimal' range through experiencing prenatal stressors. Therefore, prenatal exposure to stressors presents two avenues for impact on the resistance and susceptibility of mammals to parasites. On one hand, negative prenatal stress exposure such as bacterial/parasite infection in the mother may result in a reduction in HPA axis activity in the offspring, possibly resulting in reduced parasite resistance. However, on the other hand, stressors such as a course of LPS injections may be utilised to reduce the HPA axis activity of the offspring to assist in establishing a more efficient and optimal HPA axis responsiveness in strains.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
which are considered to have an elevated HPA axis activity. The author also notes the lack of protein related studies undertaken in this thesis. Analysis of plasma protein levels and antibody isotypes would have been pursued however due to laboratory and experimental limitations this was not possible at this time. The author also notes that all laboratory and animal work was carried out by the author only and therefore presented time and skill restraints. If the experiments in this thesis were to be repeated more emphasis would be placed on validating the qPCR results through protein analysis.

A possible role for Th17 cells in parasite immune responses

Recently, a third effector subset of CD4+ T cells has been discovered. These cells are characterised by the synthesis of IL-17, and are subsequently known as Th17 cells. Due to the synthesis of IL-17 and IL-22, Th17 cells are thought to be involved in tissue inflammation and play an important role in the immune response to extracellular parasites [56](Wen et al). As with Th1 and Th2 cells, the activity of Th17 cells can be regulated by Treg cells [56](Wen et al.). Differentiation of naive CD4+ cells relies on TGF-β and IL-6 for initial differentiation however IL-21 and IL-23 are also required for the amplification and stabilisation of differentiation respectively [57](Martinez et al.). The presence of the type 1 and type 2 cytokines IFN-γ and IL-4 suppress Th17 differentiation and an absence of IL-6 promotes TGF-β mediated Treg differentiation. Although a distinct role for Th17 cells is still uncertain, some evidence suggests that Th17 cells are involved in inflammation, more specifically neutrophil and macrophage recruitment and may play a bridging role between the innate and acquired immune responses [57,58](van der Veerdonk et al., Martinez et al.). The presence of IL-1β, a potent pro-inflammatory cytokine, in the presence of IL-6 and TGF-β enhances Th17 differentiation [57] (Martinez et al). This is of importance in this study since both IL-1β and IL-6 were shown to be upregulated locally in response to parasite infection, therefore suggesting that Th17 may play a role in the establishment of localised tissue
inflammation during parasite infection. This however was not examined due to time constraints however possess the question are Th17 cells playing a role in the initial inflammatory stage of gastrointestinal nematode infection.

Figure 5.3: Differentiation of Th17 cells. Activated APCs synthesis and release cytokines such as IL-1, IL-6 and IL-23, which promote the expansion and differentiation of Th17 memory cells to mature Th17. Release of IL-21 and IL-22 by Th17 cells further enhances amplification. Interaction with APCs and reception of IL-23 initiates release of IL-17 by Th17 cells, promoting the recruitment of neutrophils and macrophages to the infected tissue.

5.3 Closing Remarks

The project aimed to examine the role of the HPA axis in regulating the immune response to gastrointestinal nematodes. The experiments outlined in this thesis demonstrated that the action of the HPA axis affects the shape of the immune response in terms of cytokine expression. However, evidence supporting increased HPA axis activity, in terms of corticosterone production, in response to immunological stress was absent. Evidence suggested that the immune
response may be regulated by the HPA axis, where hippocampus and hypothalamus environmental monitoring was observed. Similarly, examination of the effects of parasite infection of the gastrointestinal epithelial cells suggests a role for both IL-33 and IL-25, with both cytokines demonstrating responsiveness to glucocorticoids. The basal level of HPA axis activity caused differences in the regulation of pro-inflammatory cytokines skewing the immune response towards a TH2 immune response. This was also evident at downstream sites such as the mesenteric lymph node and the spleen. Some results were also reproduced in vitro through co-culture of intestinal epithelial cells with live H. bakerii larvae, including IL-33 and IL-6 expression.

Overall, the lack of HPA axis responsiveness in terms of glucocorticoid synthesis suggests that either the immunological stressors were mild in comparison to other stressors such as restraint, heat shock etc. However, the natural basal differences in HPA axis activity between Fischer and Lewis rat strains demonstrated the ability for the HPA axis to influence the shape of the immune response. The project aimed to determine whether the HPA axis has a role in regulating the immune response to gastrointestinal parasites however evidence supporting an active role in regulating the immune response was absent. A passive role was however observed suggesting that the HPA axis may regulate the immune response as well as shaping it. The outcomes of this project have indicated a potential avenue for future experimentation addressing the issue of resistance to parasitic infection. It is believed that this thesis outlines the first documented results from H. bakerii infections in the Fischer and Lewis rat strains and from IEC6 co-culture.
Figure 5.4: The immune response overview to *H. bakerii*. Parasite infection initiates IL-33 synthesis which in turn generates the pro-inflammatory immune response (see figure 5.2 for more details). Similarly, macrophages also detect parasite infection and migrate to lymphatic centres generating the TH2 immune response through antigen presentation. Pro-inflammatory cytokines combined with macrophage antigen presentation may generate a TH17 inflammatory response early in the infection. Concurrent to this the HPA axis monitors the pro-inflammatory response/inflammatory pathology and reacts accordingly through secretion of glucocorticoids to dampen the inflammatory process. This is hypothesised to favour the generation of a TH2 immune response.
5.4 References


*O. FENNER (2012)*

231


38. Schmitz, J., et al., IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. Immunity, 2005. 23(5): p. 479-490.


43. Wills-Karp, M., et al., Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. The Journal of Experimental Medicine, 2012.


49. Coe, C.L., et al., Prenatal Stress Diminishes the Cytokine Response of Leukocytes to Endotoxin

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)


56. Wen, X., et al., Dynamics of Th17 cells and their role in Schistoma japonicum infection in C57BL/6 mice. Plos Negl Trop Dis, 2011. 5(11)


6.1 Euthanising of Rat, Tissue Isolation & Tissue Storage

6.1.1 Rat Euthanisation and Tissue Isolation & Storage
Rats were euthanised through asphyxiation, using CO$_2$ gas with the flow rate of 1L/min for 5 minutes. Following conformational tests to determine death, the chest cavity was opened and a cardiac puncture was performed using 20G needle into a 5mL EDTA vacutainer. The blood was then gently spun on a blood spinning wheel at room temperature until white blood cell isolation. The head was then removed and the brain extracted and processed. The skull was opened through use of a coping saw and pliers. The whole brain was removed as well as the pituitary gland and washed in 1x PBS. The pituitary gland was placed into a NUNC cryotube and snap-frozen in liquid nitrogen. The brain was sectioned and placed in 10% buffered formalin for histology or liquid nitrogen for qPCR. The other remaining organs where processed concurrently with the brain. The abdominal cavity was opened and the spleen removed first. The spleen was placed in PBS and spleenocytes isolated immediately. Following spleen isolation, the thymus, intestines, and adrenal glands were removed. The liver was last to be removed due to amount of blood spillage. All tissues were divided up for histology and qPCR, whilst the liver was also weighed and a portion placed into -20°C storage. Tissues isolated for qPCR were snap-frozen in liquid nitrogen in NUNC cryotubes and stored at -80°C. Tissues isolated for histology were placed into 10% buffered formalin and stored at room temperature away from sunlight.

6.1.2 White Blood Cell Isolation & Storage
Blood filled vacutainers were spun at 1500g for 10 minutes and the buffy coats were removed.
6.1.3 Spleenocyte Isolation
The spleen was removed from the animal and washed in 1x PBS solution. The washed spleen was then mashed through a cell strainer into a petri dish and the strainer rinsed into the petri dish with DMEM-10. Suspended cells were then transferred into 15mL conical tubes and spun at 800G for 3 minutes. The supernatant was discarded and the pellet resuspended in 1mL BD FACS lyse red blood cell lysis buffer. Total volume was brought to 10mL and then spun at 800G for 3 minutes. The supernatant was discarded and the pellet resuspending in 3mL DMEM-10. All steps were performed in a laminar flow hood. Cells were kept at -80°C until use.

6.2 RNA Extraction & Storage
RNA was extracted from samples stored at -80°C using the Quiagen RNesay Midi RNA Isolation Kit, following the protocol for ‘total RNA isolation from animal tissue’. Tissue excised from the animal was trimmed and placed into labelled specimen tubes and froze down in liquid nitrogen. The tissue samples were then stored at -80°C awaiting RNA extraction. At all times the sample was kept in liquid nitrogen to reduce degradation of RNA. Determination of the amount of tissue used in the RNA isolation procedure involved the weighing of the sample and specimen tube relative to an empty specimen tube. The specimen tube was then cracked open, the correct amount of tissue obtained and placed in RLT buffer for immediate homogenisation using the ‘Janke & Kunkel IKA-Labortechnik: ULTRA-TURRAX T25’ set at 24,000rpm. This procedure was then repeated for the remaining samples. The homogenised tissue was then centrifuged for 10 minutes at 3,700 rpm with no brakes. The supernatant was then removed and placed into a new centrifuge tube. To this, 1 volume of 70% DEPC-treated Ethanol was added and mixed vigorously. This mix was then immediately applied to the RNeasy midi column and centrifuged at 3,700rpm for 10 minutes. The remaining sample was
then added and spun for a further 10 minutes. If after this time the sample had not full passed through a further 10 minutes spin was applied. The column was washed with RW1 buffer before an on-column Dnase digestion was performed. The DNase solution was allowed to incubate on column for 15 minutes at room temperature before addition of RW1 buffer for a further 5 minutes incubation at room temperature. The column was then centrifuged at 3,700rpm for 10 minutes. The column was then washed with the ethanol RPE buffer and spun, with no brakes, for 15 minutes to allow the column to dry. The RNA was then eluted using RNase free water supplied in the kit.

The RNA was then stored at -80°C as an ethanol precipitate using 0.1x of NaAcetate and 2.5x of Ethanol. The ethanol-precipitated RNA was then reconstituted 48 hours later, through a 20 minute spin, followed by removal of the supernatant. Ice cold 70% ethanol was then added and further spun for 5 minutes. The supernatant was again removed and the pellet allowed to dry for 1 hour within a Bio-hood. The pellet was then resuspended in 100µl of nuclease-free water, vortexed then briefly spun in a bench top centrifuge to collect solution at bottom of tube.

The RNA was analysed for integrity using 1% agarose gel electrophoresis. The 1% gel was made up using 0.5g Sigma agarose dissolved in 50ml 0.1M TAE DEPC-treated buffer solution. 25µl of Ethidium Bromide was added to the melted solution. The gel was placed into a Bio-Rad electrophoresis tank with 0.1M TAE acting as the buffer solution. The gel was ran at 100V for 30 minutes using a Pharmacia power supply and analysed under UV light using a Syngene Transilluminator and the image captured by the GeneSnap software.

The RNA was also measured spectrophotometrically using an Eppendorf Bio-Photometer. The RNA was diluted 1:60 with 0.1M Tris.Cl DEPC-treated buffer. The readings were relative to 60µl Tris.Cl DEPC-treated buffer. The concentration of RNA was determined as well as the 260\280 reading to determine purity.

The RNA was then divided into 10µg (10,000ng) aliquots, depending on the concentrations determined by the spectrophotometer readings. The aliquots

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
were then stored at -80°C as ethanol precipitates.

6.3 Quantitative PCR

Quantitative Polymerase Chain Reaction (qPCR) refers to the contemporary method employed in measuring the level template present at the beginning of the reaction. It differs in standard PCR in that the PCR products are measured with respect to time during the entire amplification period. In this way, the rate of amplification can be integrated into enzymatic product forming rate formula resulting in a quantitative measurement of the original quantity of starting template. The qPCR reaction is frequently employed to study the level of gene expression in a given tissue or cell type.

The basic procedure for setting up qPCR assays can be summarised as:
- Primer Design
Assay Optimisation
qPCR Assay
Mathematical Quantification & Statistical Analysis

6.3.1 Two Step qPCR

Two step qPCR, as the name suggests, is a two step process in quantifying gene expression. Although more laborious and time consuming, this process is more cost effective. The process requires the separation of the reverse transcription and PCR quantification. Here RNA was extracted using the above method was quantified using the Nanodrop. Once the concentration of RNA was known, up to 5µg of total RNA was used in the reverse transcription reaction. The reverse transcription reaction was carried out as per protocol outlined in the Invitrogen SuperscriptIII first strand supermix kit. The conversion of mRNA to cDNA involved Reverse Transcriptase, and was carried out at 50°C for 10 minutes. Following cDNA synthesis, an RT denaturing step was carried out.
which involved 10 minute incubation at 85°C On completion of the cDNA synthesis reaction the cDNA was then aliquotted into 500ng aliquots at 50ng/µL and stored at -20°C. Gene expression was measured on the BioRad iCycler IQ5 using the BioRad IQ Sybr Green supermix, where cDNA was mixed with the relevant primers and Sybr green supermix. The qPCR reaction was carried out using standard denaturing, annealing and elongating steps of 95°C, 60°C and 72°C.

6.3.2 Primer Design

Primer design was carried out using the free internet program ‘PRIMER3’ (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The designing of primers for qPCR requires a number of parameters to be met. In designing primers for qPCR the amplicon was chosen so that it spanned an intron and was between 100-250bp in length. Similarly, the primers were designed so that their length was between 18 to 27 bases in length with the optimal length being 20 bases. The primers were also designed to have a Melting Temperature (Tm) of between 57°C and 63°C with an optimal Tm of 60°C. Having a number of primer sets with a similar Tm allows for the running of a number of primer sets on the same plate. For example, IL-1β, IL-6 and TNFα primers were able to be run on the same plate which reduces differing laboratory conditions between gene assays of the same subject. Primers were synthesised by Sigma Genosys Oligonucleotides and supplied as a dry pellet. The pellets were reconstituted in Nuclease-free water according to the Oligo technical data sheet, to a concentration of 100µM. An aliquot of this was taken and diluted to 10µM. From this, working concentrations were derived as 1µM concentrations to minimise repeated freeze-thaw. Reconstituted primers were stored at -20°C in clean room. The specificity of primers was verified.
through reverse transcription of RNA from target tissue, followed by PCR amplification. On both occasions gene-specific primers were used. This was then analysed on a 2% agarose gel to determine whether a single product was being formed. Alternatively, the primers were used in a one step RT-PCR using the iCyler and the melt curve used to determine a single PCR product. The PCR product was then cloned using JM109 E.coli strain. The PCR product was inserted into the Promega pGEM-T Easy plasmid vector and transformed into heat shocked calcium chloride competent JM109 E.coli cells to allow for cloning of the gene for sequencing. The sequencing procedure was carried out to verify whether the PCR amplicon was that of the targeted gene sequence.

6.3.3 Gene Fragment Cloning & Sequencing Using Transformed E.coli Cells

Rendering Cells Competent: JM109 E.coli strain bacteria were removed from -80°C and plated out on to Ampicillin-free LB agar media (see Appendix: 1.1 Solutions) and incubated for 12 hours. The plate was then stored at 4°C for 2 days. Single colonies were then inoculated into separate 15ml falcon tubes of 5ml LB broth and incubated at 37°C at a shaking speed of 250rpm for 12 hours. These tubes were then inspected and the tube with medium to high growth was selected. 4ml of this tube was then transferred to 400ml of pre-warmed LB medium and allowed to incubate at 37°C while shaking at 200rpm for approximately 3.5 hours, until an OD_{590} of 0.375 was reached. From this procedure onwards cell culture was kept cold. The culture was aliquoted equally among 4 sterilised GSA centrifuge bottles which had been kept at 4°C overnight. The culture was then allowed to cool on ice for 10 minutes. The culture was then centrifuged at 3500rpm for 7 minutes at a temperature of 4°C. The supernatant was then decanted and the cell pellet resuspended in 20ml ice-cold CaCl_{2} solution (see Appendix: 1.1 Solutions). The four bottles were then combined to form 2 bottles. These two bottles were then centrifuged for 5
minutes at 3000rpm. The supernatant was then decanted and the pellets resuspended in 40ml of ice-cold CaCl$_2$ solution. The cells were then incubated on ice for 30 minutes. The cells were then centrifuged for a further 5 minutes at 3000rpm, then resuspended in 8ml of ice-cold CaCl$_2$ solution. The cells were then dispensed as 100µl aliquots into pre-chilled 1.5ml Eppendorf tubes and stored at below -70°C.

Plasmid DNA Ligation: All reagents were taken from -20°C, and allowed to thaw on ice. The pGEM-T Easy vector and Control DNA Insert tubes were briefly centrifuged to collect all reagent at base of tube. The ligation reaction was carried out in 0.5ml centrifuge tubes and involved the addition of 5µl of 2x Rapid Ligation Buffer, 1µl pGEM-T easy Vector, and 1µl T4 Ligase with either 1µl of PCR product for the standard reaction, 2µl Control Insert DNA for the positive control and addition of milliQ H$_2$O for the negative control. All reactions were made up to a final volume of 10µl using milliQ H$_2$O. The reactions were allowed to incubate at room temperature for 1 hour before being stored at -20°C. The volume of PCR product to add to the ligation reaction was determined by establishing the concentration of PCR product using the BioRad Spectrophotometer. A 3:1 molar concentration ratio was used to determine the volume of PCR product to add.

\[
\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of insert}} \times \text{insert:vector molar ratio} = \text{ng of insert}
\]

By having values of 50ng of vector, 0.2kb size of insert, 3.0kb size of vector and a 3:1 molar ratio, it was determined that 10ng of PCR product was to be used. Therefore, 1µl was used in the reactions, since PCR products were diluted to ~10ng/µl.

Heat Shock Transformation: 25µl of rapidly thawed CaCl$_2$ competent JM109 E.coli cells were added to 2.5µl of the plasmid ligation reaction and incubated.
on ice for 10 minutes. The cells were then heat shocked involving a 40 second incubation at 42°C. 1ml of warmed Lb broth was then added and the cells incubated at 37°C for 1 hour. Aliquots were then spread onto LB\ampicillin plates and incubated for 12 hours at 37°C. The colonies were then inoculated into 1ml LB broth and incubated for 12 hours at 37°C and 300rpm. The growth solution was then separated into two separate tubes. Tube a was then pelleted through a 20 minute centrifugation, washed in 1x PBS buffer (see Appendix 1.1: solutions), and then resuspended in 50µl of 1x PBS. This solution was then boiled at 100°C for 5 minutes. This crude DNA preparation was then used as the DNA template for PCR amplification to determine whether the colonies had taken up the gene-fragment containing plasmid. Positive colonies were then grown overnight in 1.5ml LB broth in 2ml culture tubes supplied in the Eppendorf FastPlasmid Mini plasmid isolation kits.

Plasmid Isolation: Positive colonies were inoculated into 1.5ml LB broth and grown overnight at 37°C in 2ml culture tubes supplied in Eppendorf FastPlasmid Mini plasmid isolation kit. The culture was then pelleted by centrifuging the culture tubes at 14,000 rpm for 1 minute. The medium was then removed by pipetting and the culture tube allowed to drain upturned on paper. The pellet was then resuspended in 400µl of ice-cold Complete Lysis Buffer by vortexing for 30 seconds. The lysate was then incubated at room temperature for 3 minutes, before being applied to a supplied spin column assembly consisting of a spin column and collection tube. The spin column assembly was then spun at 14,000 rpm for 1 minute. 400µl of Wash Buffer was then applied to the spin column and centrifuged at 14,000 rpm for 1 minute. The follow through was then removed and the spin column assembly centrifuged for 1 minute to dry column. The spin column was then transferred to a collect tube. 50u of Elution Buffer was then added and centrifuged at 14,000 rpm for 1 minute. The spin column was then removed and the plasmid solution stored at -20°C for subsequent applications. Plasmids were then precipitated and resuspended in milliQ H$_2$O. This was then analysed through 0.7% gel electrophoresis, using quantitative HindIII digested Lambda (λ) DNA as a molecular marker.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
estimate was verified through spectrophotometry. Concentration of plasmid was essential in determining the optimal concentration of template to be added into the sequencing reaction, which was determined to be 37.5ng/µl according to protocol.

Sequencing Plasmid Insert: The plasmid DNA insert was sequenced in accordance to the CEQ 8000 protocol. The plasmid insert was cloned through a M13 mediated sequencing reaction. This involved thermal cycling of the pre-heat treatment DNA plasmid template and M13 primers, with the DTCS Quick Start Mix. The reaction mixture was then Ethanol precipitated by first mixing the sequencing reaction mixture with the stop solution. The stop solution was made into a master mix which consisted of 20µl 3M Sodium Acetate, 20µl 100mM EDTA and 10µl of kit supplied Glycogen. 2.5µl of the master mix was then mixed with the sequencing reaction before addition of 30µl of -20°C 95% Ethanol and then centrifuged at 14.1 rpm for 15 minutes. The remaining pellet was then washed in 70% Ethanol twice before vacuum drying for 1 hour. The pellet was then resuspended in 40µl Sample Loading Buffer (SLS) and allowed to sit for 15 minutes. The solution was then mixed thoroughly before being loaded onto a plate for sequencing by the CEQ 8000. The resulting sequence was then analysed using the CEQ analysis software.

6.3.4 Assay Optimisation

Optimisation of the qPCR assay is important in ensuring that the Ct values obtained are significantly accurate and that PCR amplification is 100% (80-120%) efficient. Therefore, it can be assumed that the reaction is proceeding in a logarithmic fashion. The importance of this is evident in that if two samples had the same amount of template but sample 1 had a greater efficiency; its Ct value would be less than sample 2 since a greater number of DNA strands are being generated each cycle.

Optimisation of the PCR assay involved the generation of a dilution series standard curve, which allows for the calculation of the reaction efficiency from
the slope of the curve of best fit. The generation of the standard curve involved a dilution series from 100ng to 0.01ng for low expressed genes. For higher expressed genes such as 18s the dilution series was of much lower template concentrations. Each dilution was represented on the standard curve as triplets to minimise pipetting error. The standard curve was calculated using the PCR baseline subtraction and the threshold was set manually to an acceptable level. Following this the melt curve was examined to determine whether there was a single PCR product and to determine if primer-dimer artefacts were formed.

### 6.3.5 Determination of the Reference Genes

The determination of the references genes (also referred to as housekeeper genes) is a crucial step in qPCR assay optimisation. False results may be generated by using a normalisation gene which is subject to transcription change due to the experimental treatments applied. The normalisation genes where determined for each tissue and for each differing treatments. Primer Design geNorm housekeeping gene selection kits were used to determine gene stability. A list of the normalisation genes are as follows:

- **ACTB**: β-actin
- **ATP5B**: mitochondrial ATP synthase
- **CANX**: calnexin
- **CYC1**: Cytochrome b-c1 complex subunit 4
- **UBC**: ubiquitin C
- **YWHAZ**: 14-3-3 protein/ cytosolic phospholipase A2
- **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase
- **MDH1**: malate dehydrogenase 1, NAD (soluble)
- **TOP1**: DNA topoisomerase I
- **RPL13**: 60S ribosomal protein L13
- **18s**: ribosomal subunit 18RNA
- **B2M**: beta chain of MHC class I molecules

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

243
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_StAR_F</td>
<td>CTGCTAGACCAGCCCCATGGAC</td>
</tr>
<tr>
<td>R_StAR_R</td>
<td>TGATTTCCTTGACATTTGGGTTCC</td>
</tr>
<tr>
<td>R_SF1_F</td>
<td>TCCCCCTTCTGCCGCTTCC</td>
</tr>
<tr>
<td>R_SF1_R</td>
<td>GCATTGCATCAGCACCGCA</td>
</tr>
<tr>
<td>R_FOXp3_F</td>
<td>CCCAGGAAAAGACAGCAACCTT</td>
</tr>
<tr>
<td>R_FOXp3_R</td>
<td>CTGCTTGGCAGTGCTTGAGAA</td>
</tr>
<tr>
<td>R_GR_F</td>
<td>CACCCATGATCCTGTCAGTG</td>
</tr>
<tr>
<td>R_GR_R</td>
<td>AAAGCCTCCCTCTGCTAACC</td>
</tr>
<tr>
<td>R_IL10_F</td>
<td>CACTGCTATGGCTTGCCTGCTC</td>
</tr>
<tr>
<td>R_IL10_R</td>
<td>TGTCCAGCTGGCTCTTCTTT</td>
</tr>
<tr>
<td>R_IL2_F</td>
<td>CAGCGTGTTGGATTTTGAC</td>
</tr>
<tr>
<td>R_IL2_R</td>
<td>CACAGTTGCTGGCTCATCAT</td>
</tr>
<tr>
<td>R_IL6_F</td>
<td>GCCAGAGTCATTCAAGAGCA</td>
</tr>
<tr>
<td>R_IL6_R</td>
<td>GAGCATTTGGAAGTTGGGTA</td>
</tr>
<tr>
<td>R_IFNy_F</td>
<td>GCCCTCTCTGGCTTTACTG</td>
</tr>
<tr>
<td>R_IFNy_R</td>
<td>CCAAGAGGAGGCTCTCTCT</td>
</tr>
<tr>
<td>R_TNFa_F</td>
<td>TGCCTCAGCTCTTCTTCATT</td>
</tr>
<tr>
<td>R_TNFa_R</td>
<td>GAGCCCATTTGGGAACCTTCT</td>
</tr>
<tr>
<td>R_IL1b_F</td>
<td>AGGACCCCAAGCACCTTTTT</td>
</tr>
<tr>
<td>R_IL1b_R</td>
<td>CATCATCCACAGAGTCACAG</td>
</tr>
<tr>
<td>R_P450_F</td>
<td>GCCTCCAGACTTTATTTGACTTTT</td>
</tr>
<tr>
<td>R_P450_R</td>
<td>TGGGTGTATTTCATCAGCCTTACTGAAA</td>
</tr>
<tr>
<td>R_IL1R_F</td>
<td>TGTCTACTGGAAGTGGGAATGGGTC</td>
</tr>
<tr>
<td>R_IL1R_R</td>
<td>GGGAAAGAAATCAGAGCAGAGGTC</td>
</tr>
<tr>
<td>R_CRH_F</td>
<td>CAGAACAAGAGGGCTCTCA</td>
</tr>
<tr>
<td>R_CRH_R</td>
<td>AAGGCAGAGACGGCGACAGAG</td>
</tr>
<tr>
<td>R_CRHR1_F</td>
<td>AATCCGCAGAACATCTCAG</td>
</tr>
<tr>
<td>R_CRHR1_R</td>
<td>GACACCCAGGCACACTCACC</td>
</tr>
<tr>
<td>R_POMC_F</td>
<td>TGGGTCACTTCCGCTGGG</td>
</tr>
<tr>
<td>R_POMC_R</td>
<td>TCCTCCAGCGCTCTCG</td>
</tr>
</tbody>
</table>

Table 6.1: List of primers and the respective sequences used in qPCR

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
6.4 Cortisol RadioImmunoAssays

6.4.1 Principle of Assay
The principle of a radioimmunoassay is similar to that of a competitive ELISA, instead of measuring colourometric changes, radioactivity is measured. The assay relies on the changes in the fraction of $^{125}$I corticosterone bound by the hormone specific antibody. In a sample of increased corticosterone, less $^{125}$I corticosterone is available to bind to the anti-cort antibody thus resulting in a low radioactive pellet on precipitation. However, in a sample of decreased corticosterone, more $^{125}$I corticosterone is available to bind to the anti-cort antibody resulting in a more radioactive pellet on precipitation. Therefore, the assay relies on an inverse relationship between the amount of sample corticosterone and the amount of $^{125}$I corticosterone available at precipitation.

6.4.2 Rat RadioImmunoAssay
Blood was collected from rats at given timepoints via tail vein puncture using 27G needles and 1mL syringes. The blood is immediately transferred to a 0.5mL tube containing EDTA. The tube is then completely mixed by flicking tube base with finger. Blood is then stored at room temperature whilst other rats are bled. Blood is then centrifuged at 10,000G for 5 minutes and the plasma
collected and stored in eppendorf tubes at -20°C until needed. All kit reagents (MP ImmunChem Double Anitbody, Corticosterone $^{125}$I RIA Kit) and plasma are brought to room temperature. Assay is setup as per protocol, however using 0.5x the specified amounts of plasma and reagents. Supplied quality controls are reconstituted through the addition of 2.0mL of distilled water to the relevant tubes and allowed to stand for 30 minutes. All plasma is diluted 1:200 with supplied steroid diluent where 5µl was diluted to a final volume of 1mL. Labelled Sarstedt test tubes were setup as directed in the ix: protocol. 150µL of steroid diluent was added to tubes 1 & 2. 50µL of steroid diluent was added to tubes 3 & 4. 50µL of the corticosterone calibrators (standards for generation of the standard curve) was added to tubes 5 through to 16. 50µL of the quality controls and the unknown diluted rat plasma was added to the remaining tubes. 100µL of corticosterone $^{125}$I was then added to all tubes followed by 100µL to all tubes except 1 & 2. All tubes were then vortexed and allowed to incubate at room temperature for 2 hours. Following the incubation period 250µL of precipitant solution was added to all tubes and the tubes were vortexed. All tubes were then centrifuged at 1000g for 15 minutes and the supernatants were removed via aspiration. The remaining pellet was then counted on a Perkin Elmer 2470 wizard$^2$ gamma counter using the MultiCalc software to generate the standard curve and infer the concentrations of the unknowns.

6.4.3 Sheep RadiolImmuAssay
Sheep blood plasma samples were removed from -20°C and allowed to thaw overnight at 4°C. Standards for standard curves were prepared as 0, 5, 10, 20, 75, and 500nmol/l through dilution. Similarly, the low quality control (QC) was also diluted 1:2. Tubes were labelled with tube number which corresponded to the sample outlined on the Tube map sheet. 20µl of either the standards or samples was then pipetted directly to the bottom of each corresponding tube. Tubes were then transferred to the designated radiation lab area and

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?  
N.O. FENNER (2012)
transferred to designated tube racks. 500µl of \(^{125}\)I tracer was then added to each tube and vortexed to ensure complete sample tracer mixing. Tubes were then covered and allowed to incubate at 37°C for 2 hours. Total count tubes were then removed and placed in hot area. All other tubes were then drained and heads of tubes tapped on absorbent paper. 1ml of distilled water was then added to each tube except the total count tubes. The water was then decanted from the tubes in a similar fashion as above. The tubes were then allowed to drain for 30 minutes on absorbent paper. The radioactive counts of the tubes were then measured and the samples compared to the automatically generated standard curve to give the concentration of cortisol in each sample.

## 6.5 Histology

### 6.5.1 Tissue Histology

Tissue biopsies used for histology were removed from the animal and preserved in 10% buffered formalin. Tissues were then processed for sectioning by processing through graded ethanols, xylene and then infiltrating the tissue with parafin wax (paraplast regular – Sigma) using a Miles Scientific Tissue Tek VIP system. Tissues were then embedded in parafin wax blocks using the Tissue Tek Thermal, console. Wax blocks were sectioned (5µm sections) using a Reichert-Jung microtome and placed on positively charged glass slides. For antibody staining, slides were firstly deparaffinised, rehydrated though graded alcohols to water. Antigen retrieval was carried out using Tris-EDTA pH9 solution and microwaving for 10 minutes in a 800W microwave and allowed to cool slowly and rinsed in tap water. Slides were then placed in 2.5% hydrogen peroxide in methanol for 5 minutes. Slides were then rinsed with TBS and the tissue section defined using a wax pen. The primary antibody was diluted in TBS with 0.5% BSA, 10% donkey serum and 0.025% Triton-X. Tissues were...
covered with the primary antibody and incubated at 4°C overnight. Slides were then washed with TBS-T thrice. Envision dual link (DAKO) was then applied to the slides and incubated for 1 hour at room temperature. The slides were then rinsed with TBS and DAB stained for 5 minutes at room temperature. DAB medium was then rinsed off with tap water and the slides counter stained with haematoxylin. Slides were then dehydrated and mounted in DPX medium. The slides were then viewed under a light microscopy in this case a Ziess Axio Imager.Z1

6.5.2 Immunohistochemistry

Immunohistochemistry (IHC) is a histological method employing the specificity of antibodies to visually localise a chosen antigen in tissue. Visualisation of the antigen of choice is achieved through enzymatic colour staining specific to the region where the primary antibody has recognised the chosen antigen. The process involves a number of steps similar in theory to ELISA, however IHC is less quantitative but more qualitative, distinguishing between cells in a given tissue which are expression the antigen of interest. Blocking of non-specific sites and primary antigen incubation may be used simultaneously, creating specific binding of the antibody to the antigen of choice. A secondary antibody conjugated with an enzyme is added which binds specifically to the primary antibody due to antibody isotype specificity. Addition of the substrate then creates an enzymatic reaction which allows visual localisation of the antigen in study.

6.6 Lipid Extraction from Animal Tissue

Permanently elevated glucocorticoid levels causes fat to be deposited in livers in a process known as hepatic steatosis. This is evident in human conditions such as Cushing’s disease. However, glucocorticoids are also important in liberating immune response resources such as blood glucose and protein

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
catabolism. The weights and liver contents of livers were measured to ascertain the effects of parasitism and HPA axis activity on liver fat content. The methodology used to extract total fats from tissue was based on the Folch methods. The method involved homogenisation of the tissue in 20x 2:1 chloroform-methanol with the amount being determined by the weight of the tissue used. For example, 1g of tissue would be homogenised in 20mL of 2:1 chloroform-methanol. In this instance approximately 1g of newly thawed liver tissue was homogenised in 20mL of 2:1 chloroform-methanol for 3 minutes. Homogenates were then equilibrated to room temperature. The homogenate was then filtered through fat-free filter paper. This crude extract was then mixed with 0.74% aqueous KCl, the volume being 0.2x the volume of the homogenate. The sample was then allowed to stand to separate the two phases. The upper phase was then removed and placed into pre-weighted 50mL falcon tubes. Methanol was added until solution becomes a single phase. The sample was then brought to a final volume of 50mL with the addition of 2:1 chloroform-methanol. This final volume was then allowed to evaporate at room temperature for approximately 3 days and the samples weighed at room temperature.

6.7 Heligmosomoides bakerii

6.7.1 Passaging & Culturing H. bakerii

In order to have a constant supply of fresh infective larvae ready for animal experimentation, passaging and culturing of H. bakerii had to be ongoing. A starter culture of H. bakerii larvae was obtained from Associate Professor Carol Behm at the Australian National University. These larvae were used to orally infect CBA mice. This was achieved by utilising a Gilson pipette to force 200 L3 larvae into the stomach of the mice. Faceal egg sampling occurred a week after oral dosing until positive FECs were obtained.

Once the parasite infection had been established, mice were placed on wire mesh-based cages to collect faecal matter. Faecal matter was collected into water. The faecal matter was then removed, rinsed, macerated and spread onto
the centre of a petri dish lined with filter paper (see figure 7.1). The larvae 
emerge after 5 days and were harvested by careful rinsing of the filter paper 
into a measuring flask. Larvae were washed by several rinse-settle procedures. 
The larvae were stored at 4°C in tissue flasks. Once a batch was required for 
experimentation the larvae were spun down and resuspended in sterile water. 
The larvae were separated from matter using the Baerman method and 
apparatus. The final amount of larvae were counted, spun down and 
resuspended in PBS for experimental inoculation.

6.7.2 L₃ Exsheathing

This procedure was based on the L₃ Exsheathing procedure utilised by CSIRO 
Armidale for exsheathing Trichostrongyliis colubriformis. H. bakerii larvae were 
prepared at a concentration of 1000 worms per mL in PBS. The worms were 
then treated with a 1/20 concentration of Mitton antibacterial solution (diluted 
1/20 with milliQ water) and incubated at 37°C for 20 mins. Worms allowed to 
settle before adding 20mL PBS-pen-strep. Allowed to settle and before 
repeating. Lastly, worms were resuspeded in the required cell culture media.

6.8 Standard Cell Growing Procedure

6.8.1 Standard Passaging Procedure

Rat small intestine cell lines IEC6, derived from the jejunum and IEC18 derived 
from the ileum were used in this study to be representative of the site of 
infection of H. bakerii in rats. IEC6 cells were grown cultured in high glucose 
(4500 mg/L) DMEM (Dulbecco’s Modified Eagle’s Medium – Invitrogen) 
supplemented with 5% FBS, 0.1 U/mL insulin, 50 µg/mL streptomycin and 50 
U/mL penicillin. IEC18 cells were cultured in high glucose (4500 mg/L) DMEM 
supplemented with 5% FBS, 50 µg/mL streptomycin and 50 U/mL penicillin. Cell 
cultures were grown at 37°C and 8% CO₂ taking into account the altitude at 
which the laboratory was situated (1,000m above sea level).
Cells were grown in 75cm$^2$ flasks laying horizontal. To passage cells 10mL of Tripsin (37°C) was added to media in the 75cm$^2$ flask and weas allowed to incubate for 5 mins before repeatedly pipetted to lift cells from surface. Lifted cells were then transferred to a 50mL tube with 10mL fresh media added. Cells were then pelleted by centrifugation at 200g for 5 mins. Pelleted cells resuspended in media, counted and divided into cells to be used for experimentation and passaging cells. Cells for passaging were then transferred to new 75cm$^2$ flanked and incubated.

6.9 Reagents

**DMEM-10**
DMEM-10 was used in the isolation of splenocytes and white blood cells.

1L DMEM (4.5g/L glucose, L-glutamine, sodium pyruvate)  
100mL Foetal Calf Serum  
10mL 100x PSG (penicillin G sodium, streptomycin sulphate, L-glutamine)

**DEPC treated H$_2$O**
DEPC treated H$_2$O is used in all RNA work, where the DEPC molecule inactivates RNases. Since RNases are not subject to autoclave denaturation, water is treated with DEPC, rendering the RNases inactive. The solution is then autoclaved to inactivate DEPC.

Add 1mL of 0.1% Diethylpyrocarbonate (DEPC) to 1L distilled (milliQ) water  
Mix by shaking vigorously  
Incubate overnight at 37°C  
Autoclave  
Allow to cool before use

**10x PBS (phosphate buffered saline) solution**
PBS is used for a multitude of purposes when the solution has to match the isotonic properties of cells. PBS is routinely made up in 10x stocks and stored at room temperature.
Dissolve the following in 800mL of milliQ water:

- 80g NaCl

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

251
- 2.0g KCl
- 14.4g Na$_2$HPO$_4$
- 2.4g KH$_2$PO$_4$

Adjust to pH 7.4
Bring volume to 1L
Sterilise through autoclaving

**1x PBS (phosphate buffered saline) solution**

PBS is used for a multitude of purposes when the solution has to match the isotonic properties of cells. 10x PBS stocks are diluted 1 in 10 to give a working 1x stock of similar isotonic properties of cells.

Dissolve the following in 800mL of milliQ water:
- 8g NaCl
- 0.2g KCl
- 1.44g Na$_2$HPO$_4$
- 0.24g KH$_2$PO$_4$

Adjust to pH 7.4
Bring volume to 1L
Sterilise through autoclaving

**1M Tris.Cl**

A 1M Tris.Cl stock is made up for convenience and is used in a working concentration of 10mM for nucleic acid spectrophotometry.

Dissolve 121.1g of Tris base in 800mL of milliQ H$_2$O
Bring to pH 7.4 with concentrated HCl
Bring to final volume of 1L with milliQ H$_2$O
Filter through 0.5 micron filter (optional)
Autoclave

**10x TAE buffer**

A 10x TAE buffer is made up for convenience, where a 1x working concentration is used in the preparation of agarose gels for gel electrophoresis. A 1x TAE buffer is also used as the tank buffer during the electrophoresis process.

Add the following to 800mL of milliQ H$_2$O
- 48.46g Tris base
- 3.72g EDTA-Na$_2$ salt
- 12.01g Acetic acid
Bring to 1L
Autoclave if necessary

**3M Sodium Acetate**
3M Sodium acetate is in the precipitation of nucleic acids where it neutralises the negative charge of the nucleic acid molecule allowing ethanol precipitation.

Add the following to 800mL of milliQ H\textsubscript{2}O
- 408.3g sodium acetate
- adjust to pH 5.2 with glacial acetic acid
Bring to final volume of 1L
Sterilise by autoclaving

0.5M EDTA
Add 186.1 EDTA to 800mL of milliQ H\textsubscript{2}O
Add approximately 20g sodium hydroxide (NaOH) pellets whilst stirring to bring to pH 8.0
EDTA won’t enter solution until pH 8.0 is reached
Autoclave

2% Agarose Gel Electrophoresis
Used for small fragments of DNA (such as qPCR products)
Add 1g of agarose to 50mL TAE buffer
Boil in microwave for 2 minutes until agarose is dissolved
Add ethidium bromide (10mg/mL) to a concentration of 10µg per 50mL gel
- add 1µl to 50ml gel

LB Media
Add the following to 800mL of milliQ H\textsubscript{2}O
- 5g NaCl
- 10g Tryptone
- 5g Yeast extract
- 1g Glucose
Dissolve ingredients and bring to final volume of 1L
Autoclave
Add the following if desired:
- Ampicillin to 50µg/mL
- Tetracycline to 12µg/mL
- Xgal to 20µg/mL
- IPTG to 0.1mM

LB Plates
Add the following to 800mL of milliQ H\textsubscript{2}O
- 5g NaCl
- 10g Tryptone
- 5g Yeast extract
- 1g Glucose
- 15g Agarose
Dissolve ingredients and bring to final volume of 1L

Add the following if desired:
- Ampicillin to 50µg/mL
- Tetracycline to 12µg/mL
- Xgal to 20µg/mL
- IPTG to 0.1mM

CaCl₂ solution
CaCl₂ is used in the preparation of competent cells, and is used ice cold.

60mM CaCl₂ (1.76g/200mL solution)
15% glycerol (30mL/200mL solution)
10mM PIPES [piperazine-N,N'-bis(2-hydroxypropanesulphonic acid)]
(0.6g/200mL solution)
Bring to pH7.0
Autoclave
Store at room temperature and use ice cold

Ficoll-Hypaque Solution
Ficoll-Hypaque solution is used to separate PBMCs from erythrocytes and plasma by creating a density gradient when centrifugation is applied.

Dissolve 6.2g Ficoll in 75mL distilled water with slow stirring.
Add 10.4g sodium diatrizoate and stir until solution is clear
Bring final volume to 100mL
Filter sterilise using 0.22µm filter
Store for up to 6 months at -4°C in dark

Neutralisation buffer
1.1g NaHCO₃
0.1g NaOH ~ 1 pellet
10mL of 1M HEPES
Make to 50mL with milliQ water and filter sterilise

Pure Upper Phase
Mix chloroform, methanol and 0.74%KCl in a ratio of 8:4:3, and mix in a separation funnel. Allow mixture to stand separating the two phases. The lower phase contains chloroform, methanol, and water in the ratio of 86:14:1, whilst the lower phase is in the ratio of 3:48:47.

IEC6 Cell Media
Glucose (4500 mg/L)
DMEM (Dulbecco’s Modified Eagle’s Medium – Invitrogen)

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
5% FBS
0.1 U/mL insulin
50 µg/mL streptomycin
50 U/mL penicillin

**IEC18 Cell Media**
Glucose 4500 mg/L
DMEM
5% FBS
50 µg/mL streptomycin
50 U/mL penicillin

7.1 Introduction

This chapter contains various pilots experiments and experiments that supplemented data from previously published data that was unable to provide enough data to constitute a full chapter. Due to time constraints these avenues of investigation were not fully completed, however if time permitted would have been investigated further to develop further chapters investigating the role of the neuroendocrine system in regulating the immune response to gastrointestinal nematodes.

7.1.1 Background: Sheep Experiments

As with rats, sheep are susceptible to parasitism by gastrointestinal nematodes. Of agricultural importance *Haemonchus contortus* and *Trichostrongylus colubriformis* stand out as particularly significance parasites, with overall cost to the industry of over AU$400 million per annum contributed to ovine gastrointestinal parasitism.

As with the immune response seen in rats in response to gastrointestinal parasitism, sheep also exhibit marked changes in immune responsiveness to parasitism. Prolonged infestation can cause body condition loss, dehydration, anaemia and in severe cases death may occur. In order to circumvent these losses various anthelmintics, in the form of drenches have traditionally been utilised. However, due to the rise and development of anthelmintic resistance new approaches are been sought to prevent these industry losses. Natural resistance against gastrointestinal nemaode infection has diminished as more emphasis is placed on productivity based traits such as wool and meat characteristics. However, as with most parasitic infections resistance to infection depends on the mounting of a Th2 immune response. Conversely, susceptibility may be characterised by the mounting a Th1 immune response, reducing the ability of lambs to clear infection.
An animal trial was conducted, with the aim to differentiate genes important in conveying resistance and susceptibility to *H. contortus* and *T. colubriformis*. As well as examining the primary response to these parasites, trials were also conducted to simulate the acquired immune response through multiple separate infections. By doing this the researchers were able to differentiate between genes important in the innate and acquired immune response to *H. contortus* and *T. conlubriformis* separately [1,2].

If we look at the *T. colubriformis* challenge by examining gene expression in jejunal tissues, it is evident that susceptible sheep may be slower in mounting an inflammatory response during the innate period, where IL-12β, NFκB1B and IkBKB are higher, however GATA3 and IFNγ are reduced compared to the resistant line [1,2]. This suggests that the resistant line is able to respond faster to infection in the innate period conveying resistance, and not differential gene expression. Interestingly, at the same timepoint sheep resistant to *H. contortus* infection, infected with *T. colubrifoirmis*, showed a similar response in jejunal tissues, where IL-1β expression was lower in susceptible sheep but NFκB1A expression was higher. When *H. contortus* infections are examined a similar response is observed, with sheep resistant to *T. colubriformis* infection mounting a strong inflammation response in the abomasum, with increased IFNγ and IL-10 expression during the innate response. This poses the question “are sheep that have been selected for resistance against *T. colubriformis* infection also resistant to *H. contortus* infection and vice versa?”. Examining the FEC data suggests that resistance to one parasite also conveys protection against the other parasite, however this was not always evident until secondary infections had occurred suggesting that the inherited phenotype in resistance involves mechanisms involved in the acquired and not innate immune responses.

With both infections, sheep resistant to the particular parasite mounted a strong IL-2R response in the infected tissue, but not in unaffected tissues [2]. Confirming that a strong Th2 response is crucial in conveying resistance against sheep parasites.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER* (2012)
7.1.2 Background: Rat Histology
The HPA axis functions to regulate and control the synthesis of glucocorticoids by monitoring inflammation and reacting accordingly. Detection of inflammation, possibly through detection of systemic IL-1β and activation of localised nerve signals, causes CRH and AVP to be synthesised at the parvocellular cells of the paraventricular hypothalamic nucleus and secreted into the median eminence though axonial transport to be detected by corticotrophic cells of the anterior pituitary. This chapter will show preliminary tissue histology results showing the localisation of IL-1β and IL-1R1 protein in the rat brain. Previously, in chapter 3, it was demonstrated that the expression of both IL-1β and IL-1R1 are upregulated in response to gastrointestinal nematodes. The expression of IL-1β and IL-1R1 was shown to be upregulated in the hypothalamus in response to infection, however only IL-1β was upregulated in the hippocampus. This chapter will examine the localisation of IL-1β and IL-1R1 protein in brain sections using immunohistology techniques.

7.2 Materials and Methods
Details of the sheep trials utilised in this chapter have been published previously [1,2]. Details of the rat trials utilised in this experiment can be found in chapter 3.
7.3 Results

7.3.1 Pro-Inflammatory cytokine expression in the jejunum and abomasum in response to gastrointestinal nematode infections

Interleukin-1β expression in response to parasite infection

The upregulation of IL-1β in tissue is indicative of inflammation and is important mediator of pro-inflammation locally as well as signalling inflammation to the neuroendocrine system. Tertiary *H. contortus* infection increased IL-1β in the abomasum but not the jejunum. Tertiary *T. colubriformis* infection had no effect on IL-1β expression. A mixed primary infection of both parasites had no effect on expression of IL-1β in either the abomasum or jejunum. IL-1β expression was upregulated 4.5 fold compared to control group (p=0.02). The tertiary response to *H. contortus* was 4.3 fold higher in IL-1β compared to the innate response (p=0.02).

![Figure 1: IL-1β expression in the parasitised abomasum](image)

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

*N.O. FENNER (2012)*
Figure 2: IL-1β expression in the parasitised jejunum. There was no significant changes in IL-1β expression in response to parasite infection.

Tumour Necrosis Factor α expresion in response to parasite infections
As with IL-1β, TNFα is also an important mediator of local inflammation and may also initiate signalling to the neuroendocrine system. Tertiary *T. colubriformis* infections decreased TNFα expression in the jejunum but not the abomasum. Tertiary *H. contortus* infection had no effect on TNFα expression. Mixed primary infection had no effect on TNFα in either the abomasum nor the jejunum. TNFα expression was downregulated by 2.2 fold in response to tertiary *T. colubriformis* infection compared to control (p=0.01).
Figure 3: TNFα expression in the parasitised abomasum. There was no significant changes in TNFα gene expression in response to parasite infection.
Figure 4: TNFα expression in the parasitised jejunum. Expression of TNFα was down regulated in response to tertiary T. colubriformis infection.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
7.3.2 Expression of key genes of the cortisol producing pathway in response to gastrointestinal nematodes in sheep selectively bred to display resistance or susceptibility to *T. colubriformis*

A flock of sheep (TSF), selectively bred to display resistance or susceptibility to *T. colubriformis*, were given either primary or tertiary infections of either *T. colubriformis* or *H. contortus* and subsequent changes in gene expression in the adrenal gland were measured. Of the genes measured MC2R, SF-1 and StAR showed significant differences. Of the two parasites, it was *H. contortus* that generated the most significant response bearing in mind that the flock was selected for responsiveness to *T. colubriformis*. This suggests that in terms of stress defined by HPA axis responsiveness that *H. contortus* infections are more significant than *T. colubriformis* infections.

The MC2R is a receptor involved in the HPA axis signalling where ATCH is received by the adrenal gland. Reception of ATCH by MC2R initiates PKA activation, which promotes the transcription of genes involved in cortisol synthesis. In this experiment, there was a significant line x treatment interaction (*p*=0.004) for *H. contortus* infections, whereby resistant TSF sheep demonstrated higher levels of adrenal MC2R expression in the primary infection compared to the tertiary infection (figure 7). However, the opposite was observed for the susceptible TSF sheep, where MC2R expression was higher during the tertiary response compared to the primary response. Infection of TSF by *T. colubriformis* had no effect on MC2R expression.

Reception of ACTH by MC2R results in activation of PKA which phosphorylates the transcription factor SF-1. In this experiment the expression of SF-1 was found to be greatest in the *H. contortus* infected group compared to the *T. colubriformis* group (*p*=0.026) (figure 8). There was no difference in SF-1 expression between treatments within these groups, nor was there any difference in terms of resistance and susceptibility.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*

263
The protein StAR is an important factor determining the cholesterol availability, promoting intra-mitochondrial cholesterol transfer in adrenal cortex cells. The StAR promoter sequence has several sites for transcriptional factor recognition including SF-1 and therefore reception of ACTH is related to StAR transcription through transcription factor activation including SF-1. Sheep infected with *H. contortus* demonstrated higher levels of StAR expression than sheep infected with *T. colubriformis* (p=0.032) (figure 9). This mirrors expression trends seen with SF-1, providing evidence towards the link between SF-1 and StAR expression. Infection by either parasite induced higher levels of StAR expression in susceptible sheep during the tertiary infection compared to the primary infection. However, resistant sheep had higher levels of StAR expression during the primary infection compared to the tertiary infection (p=0.012), which was a similar trend seen with MC2R expression.
Figure 5: CYP11A1 expression in the adrenal gland in response to gastrointestinal nematode infection. The graph shows the expression levels of CYP11A1 in the adrenal gland of TSF sheep infected with either *T. colubriformis* (blue bars) or *H. contortus* (red bars) compared to naïve station sheep as controls (green bars). SEM is represented by error bars. There were no significant interactions.
Figure 6: IL-1β expression in the adrenal gland in response to gastrointestinal nematode infection. The graph shows the expression levels of IL-1β in the adrenal gland of TSF sheep infected with either *T. colubriformis* (blue bars) or *H. contortus* (red bars) compared to naïve station sheep as controls (green bars). SEM is represented by error bars. There were no significant interactions.
Figure 7: MC2R expression in the adrenal gland in response to gastrointestinal nematode infection. The graph shows the expression levels of MC2R in the adrenal gland of TSF sheep infected with either *T. colubriformis* (blue bars) or *H. contortus* (red bars) compared to naive station sheep as controls (green bars). SEM is represented by error bars. TSF resistant sheep expressed more MC2R during the tertiary *H. contortus* infection compared to the primary infection. TSF susceptible sheep expressed more MC2R during the primary *H. contortus* infection compared to the tertiary infection.

*Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?*

*N.O. FENNER (2012)*
Figure 8: SF-1 expression in the adrenal gland in response to gastrointestinal nematode infection. The graph shows the expression levels of IL-1β in the adrenal gland of TSF sheep infected with either *T. colubriformis* (blue bars) or *H. contortus* (red bars) compared to naïve station sheep as controls (green bars). SEM is represented by error bars. Overall expression of SF-1 was higher in the response to *H. contortus* compared to *T. colubriformis*. Line and treatment had no significant effect.
Figure 9: StAR expression in the adrenal gland in response to gastrointestinal nematode infection. The graph shows the expression levels of StAR in the adrenal gland of TSF sheep infected with either *T. colubriformis* (blue bars) or *H. contortus* (red bars) compared to naïve station sheep as controls (green bars). SEM is represented by error bars. *H. contortus* infected sheep had higher levels of StAR expression compared to *T. colubriformis*. TSF resistant sheep expressed more StAR during the tertiary *H. contortus* infection compared to the primary infection. TSF susceptible sheep expressed more StAR during the primary *H. contortus* infection compared to the tertiary infection.
7.3.3 Rat Brain Histology

Histological samples were taken at time of autopsy, whereby brain sections were dissected and stored in formaldehyde solution outlined in the appendix. These samples were hoped to be used to confirm qPCR results outlined in the chapter 3 where expression of IL-1β and IL-1R1 were measured in the hippocampus and hypothalamus in response to gastrointestinal nematode infection. The results showed that both IL-1β and IL-1R1 were upregulated in the hypothalamic tissue while IL-1β only was upregulated in the hippocampal tissue. The following results outline a truncated experiment to confirm these results using immunohistology. Results demonstrated that both IL-1β and IL-1R1 protein were present in parasitised rat brain tissue. The location of IL-1β suggests that the protein was located in both the hypothalamic and hippocampal tissue, as suggested by qPCR results. The location of IL-1R1, however seems to be restricted to the median eminence. There was little evidence seen of IL-1R1 protein in the hippocampal tissue. Although needed to be confirmed by a specialist in brain anatomy, localisation of IL-1β is restricted to the lateral hypothalamus & ventromedial thalamic region, the lateral posterior thalamic nucleus medial amygdaloid nucleus. There was no positive staining recorded for the isotype control.
Figure 10: Localisation of IL-1β protein in the lateral hypothalamus & ventromedial thalamic region
Figure 11: Localisation of IL-1β protein in the lateral posterior thalamic nucleus
Figure 12: Localisation of IL-1β protein in the medial amygdaloid nucleus
Figure 13: Localisation of IL-1R1 protein in the median eminence.
Figure 14: Localisation of IL-1R1 protein in the median eminence.
Figure 15: Isotype control.
7.4 Conclusion

Although time and resources did not permit further investigation of the above results, some interesting observation were made which may present further avenues of investigation in the future. We showed that pro-inflammatory cytokines were differentially expressed in response to parasite infection, we showed that key cortisol synthesis genes were differentially expressed in response to parasite infection and that expression differed dependant on host's responsiveness, and we also showed localisation of IL-1β and IL-1R1 in the brain which complemented work done in chapter.

7.4.1 Expression of pro-inflammatory cytokines in gastrointestinal tissue in response to parasite infection

The expression of IL-1β and TNFα was measured in sheep gastrointestinal tissues after being subject to either a single mixed infection (innate) or multiple distinct single parasite infection (acquired). This design was aimed to examine the differences in gene expression for innate and acquired immune responses and also to determine whether the immune response to both parasites was different. The results showed that in the abomasal tissue *H. contortus* infection increased IL-1β expression only during the acquired response. Similarly, for an acquired *T. colubriformis* infection TNFα expression was increased in the jejunum only. This demonstrated that expression of pro-inflammatory cytokines was parasite specific, with site of infection being a major factor. The results demonstrated that gastrointestinal nematode infection in sheep induces pro-inflammatory gene expression in the parasitised tissue dependant on parasite species and whether the infection was a primary or tertiary infection.

7.4.2 Expression of key genes of the cortisol synthesis pathway in high and low responding sheep in response to parasite infection

The expression of a number of key genes of the cortisol synthesis pathway was
measured in the adrenal glands of sheep selected for either high or low responsiveness to *T. colubriformis* infection (TSF). These high and low responding TSF sheep were denoted as either resistant (high responsiveness) or susceptible (low responsiveness) to *T. colubriformis* infection. Both resistant and susceptible TSF sheep were infected with either a primary (innate) or tertiary (acquired) infection of either *T. colubriformis* or *H. contortus*. This experiment complements other work [1,2] which utilised the same sheep trial. Overall, the results demonstrated that gastrointestinal nematode infection in sheep induces cortisol synthesis gene expression changes in adrenal tissue. Similarly, it also apparent that there is gene expression differences between resistant and susceptible in response to infection. There were no significant differences in the expression of CYP11A1 and IL-1β in response to parasite infection in adrenal tissue. However, genes MC2R, SF-1 and StAR did show significant differences. Reception of ACTH through MC2R initiates a signalling cascade resulting in activation of PKA. Phosphorylation of transcriptional factor SF-1 is achieved via activity of activated PKA. Phosphorylated SF-1 promotes the transcription of certain genes involved in the stress response including StAR.

*H. contortus* infection affected the expression levels of MC2R, where resistant TSF had higher levels of MC2R expression than during the tertiary infection. However, the opposite was observed with the susceptible TSF whereby expression of MC2R was higher at the tertiary infection compared to the primary infection. A similar pattern of gene expression was observed with StAR expression being higher during the tertiary infection in susceptible TSF but higher in resistant TSF during the primary infection. This suggests that a strong adrenal response during an innate infection may be important in directing an effective immune response. Similarly, it also may involve gene expression timing, since only a single timepoint in the infections (day 3 of a primary or tertiary infection) was examined. Therefore, it is unsure whether gene expression of MC2R and StAR occurred earlier or later during the infection, however what is know is that timing between susceptible and resistant TSF is
different and that this may present a selective mechanism, however further investigation would be required. Also the TSF sheep used in the experiment were selectively bred for susceptible or resistance to *T. colubriformis* there was no change in expression of key cortisol synthesis genes in response to *T. colubriformis* infection. The only differences found were observed during TSF infections with *H. contortus*, suggesting that the tissue damage inflicted by *H. contortus* infection initiates a higher HPA axis response than *T. colubriformis*. This is evident in the expression of SF-1, where *H. contortus* infection induced significantly higher levels of expression compared to *T. colubriformis* infection.

7.4.3 Localisation of IL-1β and IL-1R1 in the parasitised rat brain

By taking histological brain sections at the time of autopsy, significant genes expressed in the hippocampus and hypothalamus were able to be localised. Of the genes examined in the brain two genes of importance became evident. It was found that infection of rats with *H. bakerii* induced gene expression changes in the hippocampus and hypothalamus (chapter 3). In the hippocampus IL-1β expression was upregulated in response to parasite infection by 436% in fischer and 160% in lewis. In the hypothalamus IL-1β expression was upregulated by 217% in fischer and 209% in lewis in response to parasite infection. These qPCR results were some what confirmed using histology, where the histology tissue sampled localised IL-1β to both hippocampal and hypothalamic tissues as measured in qPCR results. IL-1R1 was also measured in qPCR results with a 56% increase in expression in fischer and 74% increase in expression in lewis. This result was also some what confirmed with histology localising IL-1R1 protein to the median eminence region of the brain, a section that was included in the hypothalamic tissues used in qPCR measurements. Taken together, these results present evidence that IL-1β signalling is important in conveying messages to the brain, utilising both the hypothalamus and the hippocampus. Localisation of IL-1R1 at the median eminence suggests that IL-1β messages are able to be translated to the brain.
without the need for IL-1β protein to cross the blood-brain barrier. Similarly, this also suggests that systemic IL-1β levels in the blood may play an important role in signalling stress to the brain.

7.5 References


CHAPTER 8: Posters

Gastrointestinal Nematode Infection Induces Changes in HPA Axis Gene Expression

NEAL FENNER1, DEBORAH HODGSON2 & PETER HUNT1
1 CSIRO Livestock Industries, QLD Master Laboratory, Locked Bag 1, Armidale 2350, NSW Australia
2 Laboratory of Neuroimmunology, School of Psychology, University of Newcastle, Newcastle 2308, NSW Australia

Introduction
The Hypothalamus-Pituitary-Adrenal (HPA) axis mediates the synthesis of glucocorticoids in response to stress. Recent research also suggests that glucocorticoids influence the immune response, inhibiting the TH1 response, allowing TH2 responses to become more evident.

Key Points:
- Gastrointestinal Nematodes Induce a TH2 bias
- Glucocorticoids inhibit TH1, favouring TH2 immune responses
- Therefore, the HPA Axis may play a crucial role in the regulation of the immune response to Gastrointestinal Nematodes

Sheep Experiment
Outbred merino sheep were artificially infected with either Trichostrongylus colubriformis or Haemonchus contortus, repeatedly (acquired response) or with T. colubriformis and H. contortus once (innate response).

- Treatment Groups -
  - **Control**: Helminth naive
  - **Innate**: Single mixed species infection three days prior to slaughter
  - **Acute**: Four T. colubriformis infections
  - **Chronic**: Four H. contortus infections

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genes Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrointestinal Tract</td>
<td>IL-1α, TNF-α, IL-10, VCAM</td>
</tr>
<tr>
<td>Adrenal Gland</td>
<td>CYP11α1, MC2R, SBAR, SP1</td>
</tr>
<tr>
<td>Pituitary Gland</td>
<td>CRH, PCNA, POMC, POMC, AVP</td>
</tr>
</tbody>
</table>

Conclusion
It was evident from this study that the HPA axis is involved in regulating the immune response to gastrointestinal nematodes, possibly stimulating via systemic pro-inflammatory cytokines synthesised at the site of infection. To further investigate the role of the HPA axis in regulating the TH2 response, a rat model is currently being developed utilising the inherent differences in HPA axis activities of the Lewis and Fischer rat strains in the context of the immune response.

Does the Hypothalamus-Pituitary-Adrenal Axis Play a Role in the Immune Response to Gastrointestinal Nematodes?

N.O. FENNER (2012)
Introduction
The Fischer and Lewis rat strains differ in Hypothalamus-Pituitary-Adrenal (HPA) axis responses to stress. Lewis rats secrete little corticosterone in response to stress and are susceptible to inflammation whilst Fischer rats secrete larger quantities of corticosterone and are inflammation resistant. 

The immune response to gastrointestinal nematodes is classically TH2, characterised by type 2 cytokine production, eosinophilia and antibody class switching, and stimulated by glucocorticoids. This study aimed to discover whether Fischer and Lewis rats mount differing immune responses to infection with the parasitic gastrointestinal nematode Heligmosomoides bakeri due to their differing HPA axis activity. Rats were studied as they are the model organism for neuroendocrine study, whilst the particular parasite was chosen due to similarity of life cycle with livestock parasites.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISCHER parasite</td>
<td>Oral inoculation: 4,000 L3 H. bakeri larvae</td>
</tr>
<tr>
<td>LEWIS control</td>
<td>Saline</td>
</tr>
<tr>
<td>LEWIS parasite</td>
<td>Oral inoculation: 4,000 L3 H. bakeri larvae</td>
</tr>
</tbody>
</table>

Results
HPA Axis Gene Expression:

Conclusion
The results showed that there were significant differences in the immune responses of Fischer and Lewis rats to H. bakeri. Interestingly, IL-10 responses were evident in neuroendocrine, but not immune tissue. IL-6 gene expression however was altered in response to infection in immune/hypothalamic tissue. Analysis of lymphoid tissue showed up-regulation of the TH2 cytokines IL-10 and IL-13 and down-regulation of the TH1 transcription factor STAT4. In response to infection, IL-10 up-regulation was greater in Lewis than Fischer rats, whilst up-regulation of IL-13 occurred similarly in the two strains. STAT4 was down-regulated in response to infection in both rat strains, but the degree of down-regulation was more substantial in Lewis rats. These results coupled with the fact that corticosterone levels were not significantly increased due to parasitism suggest that basal levels of HPA axis activity rather than HPA axis responsiveness is important in the immune response of rats to H. bakeri.

Methodology
Fischer and Lewis rats were inoculated with 4,000 H. bakeri L3 larvae. HPA axis and immune tissues were harvested at day 10 post-inoculated and HPA extracted. Rats were chosen as host as they are model organisms for neuroendocrine study. H. bakeri was chosen as a homologous parasite to classical gastrointestinal nematodes found in livestock.