Final report

Immune fitness as a measure of animal health, welfare and productivity

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Abstract

This project aimed to provide an objective measure of an animal's immune fitness, otherwise termed immunocompetence. Immune fitness enables production animals to respond to challenges affecting health, well-being and productivity. Such a measure would assist industry stakeholders to evaluate the positive impact of changed management procedures and to support productivity ("fit for purpose"). Biological samples sourced from sheep and cattle exposed to pathogens and stressors relevant to the Australian red meat industry were analysed by a wide range of techniques including transcriptomics (gene expression i.e. which genes are switched 'on' or 'off'), proteomics (proteins produced in response to immune stimulants), telomere analysis ('biological age'), serum trace mineral assessment and determination of stress markers (cortisol), to identify putative biomarkers of immune fitness. The key findings from the study revealed panels of genes that were differentially expressed and telomere length estimation, that at an early stage in the production process in both sheep and cattle were predictive of production outcomes (disease resilience and/or capacity to achieve weight gain). These markers are suggested as candidates for creation of a panel for production scoring termed the Immune fitness Index. This index would enable producers to identify cattle or sheep at risk of failure to thrive, triggering adaptations to management for improved animal welfare. It could also be adopted as a marketable commodity at point-of-sale, as a measurable index of the health and welfare of the animals through the production chain. Further, the research has identified genes associated with a baseline for cattle resulting in meat scored within the top 25% MSA index. Consideration of health, welfare and production aspects may enable the selection of high performing livestock without compromising the health and welfare of the animal that would have foreseeable financial benefits to the producer and would aid in adoption of the Immune fitness index.

Executive summary

Background

As global consumption of red meat, and in particular beef, continues to rise, Australia remains one of the top global exporters or red meat and live animals (DOAWE, 2021; PWC, 2011). The red meat and livestock industry has grown by 42% from 2013-14 to 2018-19, driven in part by an increasing shift towards the feedlot sector to satisfy export demands in an increasing drought- and flood-stricken environment. The feedlot industry turnover increased from $2,787million to $4,635million in the same time period (Packer, 2020). Meeting increasing demand in the face of an adapting global climate necessitates streamlining of production processes to ensure high production outcomes while also optimising livestock health and welfare experiences. This calls for development and integration of novel methods that seek to facilitate the best for both livestock and producer. This project aimed to provide an objective measure of an animal's immune fitness, otherwise termed immunocompetence. Immune fitness enables production animals to respond to challenges affecting health, well-being and productivity. Such a measure would assist industry stakeholders to evaluate the positive impact of changed management procedures and to support productivity ("fit for purpose") with a view to achieving optimal production outcomes.

The project objectives were three-fold. Firstly, we aimed to develop a prototype scorecard to categorise animal immune fitness comprising of: (i) a suite of immunological tests for identifying immune factors that may affect the welfare and health of animals, which are impacted by production processes, and (ii) genetic correlates of infection. The second objective was the identification of critical pressure points impacting immune fitness along the production chain and thus identification of areas of risk to welfare and productivity which will enable integration of welfare through the value chain. Thirdly, we aimed to identify and validate a baseline for immune fitness linked to red meat quality and assess parameters in association with health, disease, and production outcomes.

Methodology

Sheep and cattle samples were sourced from experimental pathogen exposure trials, commercial farms and feedlots. Collected samples were processed for a range of outcomes as illustrated in the Fig. 1 below.
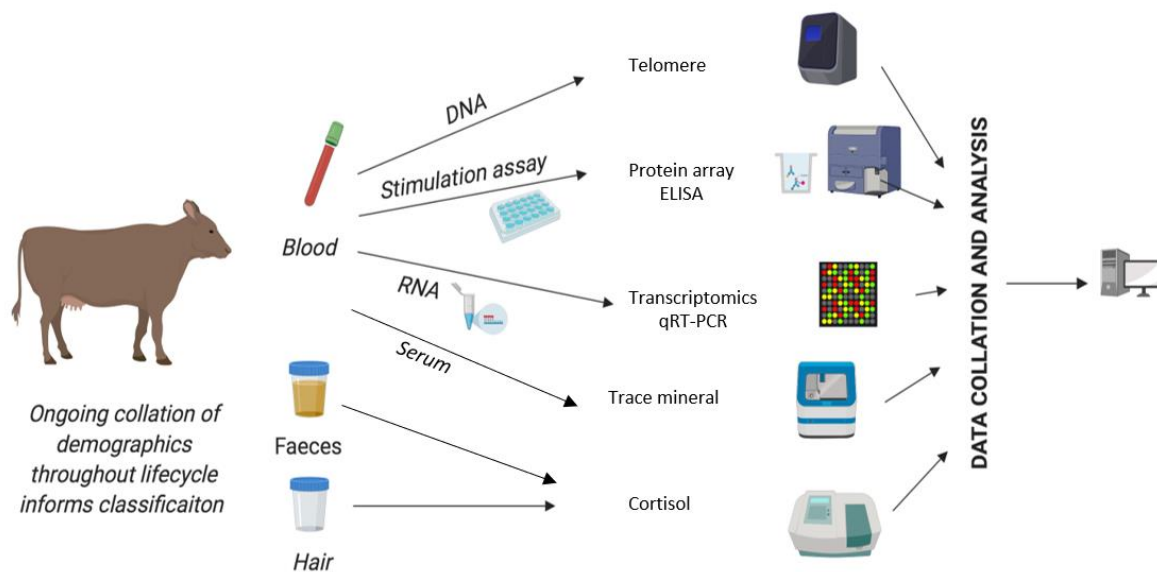
Figure 1: Experimental model for dissecting immune response variability in sheep and cattle. Biological samples sourced from sheep and cattle exposed to pathogens and stressors relevant to the Australian red meat industry were analysed by a wide range of techniques including Telomere analysis ('biological age'), Proteomics (protein arrays and ELISAs to detect proteins produced in response to immune stimulants), Transcriptomics and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for gene expression (i.e. which genes are switched 'on' or 'off'), serum trace mineral assessment and determination of stress markers (cortisol), to identify putative biomarkers of immune fitness.

Results/key findings

The first objective was met through the identification of correlates of immune fitness that are reported in this final report. These are suitable to take forward to the development of a panel for inclusion to a score card and the development of an '**Immune Fitness Index**' similar to the Meat Standards Australia (MSA) index. Our second objective in relation to critical pressure points was assessed using a novel approach. A meta-analysis of transcriptomic datasets sourced from research carried out on cattle exposed to a range of stressors (detailed below) was conducted and this enabled identification of key pressure points associated with the capacity of beef cattle to achieve optimal production outcomes. Our early investigation revealed a paucity in transcriptomic analysis of feedlot finished cattle exposed to environmental stressors thus we carried out an analysis of cattle progressing through feedlot (short finishing). This combined with data sourced following a meta-analysis facilitated identification of baseline expression of genes associated with optimal red meat quality, aligned with cattle achieving the top 25% MSA index score, thus addressing the third project objective.

Details of the key findings are below:

- Meta-analysis of seventeen transcriptomic datasets encapsulating the gene expression responses of sheep or cattle exposed to the pathogens *Brucella ovis* (sheep), *Mycobacterium bovis* (cattle), *Mycobacterium avium* subsp. *paratuberculosis* (sheep and cattle), and *Rhipicephalus microplus* (cattle), Pestivirus (cattle), *Neotyphodium coenophialum* (cattle), and acidosis revealed 165 genes meeting the criteria for differential expression associated with disease resilience in cattle across multiple breeds, sample sources and disease exposure states, similarly, 1437 differentially expressed genes are significantly regulated in sheep achieving resilience to disease. Selections of these genes were validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

- Combinations of the above genes would be suitable for inclusion into a panel (scorecard) to determine which animals have the best resilience to disease burden and/or higher immune fitness.

- Fifty-four genes meet the criteria for differential expression for cattle achieving both high average daily gain (ADG) and a high MSA index score. These could be utilised as correlates of production outcome associated with immune fitness in feedlot cattle.

- Meta-analysis of public sourced transcriptomic databases combined with transcriptomic data sourced from cattle undergoing production stressors enabled in-depth comparison between multiple environmental stressors with production outcomes (ADG, MSA index score and disease status) and identified adaptation to variation in nutrient source as a primary pressure point in determining the capacity of an animal to achieve optimal production outcomes.

- We have identified 226 genes meeting the criteria for differential expression either at induction of cattle to feedlot or consistently across both sampling times (Day 0 and day 76) that are associated to cattle achieving a high MSA index score in comparison to those who achieved low score. The expression of these genes may serve as a baseline for optimal red meat quality metrics regardless of breed, farm of origin and exposure to environmental stress.

- Telomere length could be utilised as a biomarker to determine which animals have the best resilience to disease burden and higher immune fitness. Relative telomere length could additionally be used to determine best practice for livestock production.

- The protein array identified putative biomarkers for immune fitness, however, the technology to adopt these using cost-effective testing is not currently available. At this time, we do not recommend inclusion of these in the immune fitness scorecard.

- Cortisol (hair and faeces) and trace minerals were not strongly associated with immune or production outcomes and are therefore not recommended for inclusion in the immune fitness scorecard.

Benefits to industry

We have identified quantifiable measures of sustained health and welfare through delivery of a prototype scorecard for immune fitness. This provides an opportunity for the livestock industry to look holistically and address drivers that can affect productivity, regardless of production pressures that may be specific to a particular producer.

Key benefits foreseen include:

- Enabling adaptation of handling and management strategies through application at pressure points, leading to improved animal welfare and economic benefits to the red meat industry.
- The **'Immune Fitness Index'** based on the panel of biomarkers included in the scorecard could serve as a marketable commodity at point of sale, with benefits to both the producer and the consumer.
- The biomarkers would facilitate identification of animals at risk of failure to thrive throughout the production chain and would be valuable to use from farm to post farm-gate.
- The **'Immune Fitness Index'** could be utilised to increase public confidence and improve perceptions around animal health and welfare in the livestock industries. This graded index could provide reassurance to consumers that animal welfare has not been compromised along the production chain. Working collaboratively with farmers and researchers assessing animal wellbeing through the lifespan of the animal, this can enable a quantifiable assessment of the health status and productivity of livestock.
- There were specific biomarkers associated with meat quality, many of which were aligned to immune fitness. These could be easily adopted by producers to facilitate selection of high performing livestock without compromising the health and welfare of the animal. This would have foreseeable financial benefits to the producer and would aid in adoption of the **Immune fitness index**.

Future research and recommendations

Working together with MLA, Industry and other livestock scientists, we propose further validation prior to adoption of the **Immune Fitness Index**. This should include validation of the relevance of these biomarkers across breeds and production systems. Further, the gene expression signature(s) identified as predictors of immune fitness and MSA Index could be incorporated into existing EBVs.

We have identified a putative farm-of-origin effect that is not explainable by breed or sex variations. This suggests environmental factors potentially impacting on internal biology, such as the diet, soil type, or stress they experience. The latter can be associated with animal husbandry and minimised by awareness and empathy of stock hands regarding humane practices. Furthermore, the microbiome (gut microbiota), which has co-evolved with animals and encodes many important metabolic functions as well as interacting with the immune system, is dynamic and can also be impacted by environmental and dietary factors. As beef cattle move through a feedlot system their feed ration changes to a finishing diet with a higher proportion of grain compared to their pasture-based diet on farm. Consequently, the microbes within the rumen of these cattle must adapt to extract energy from the high concentrate feed as opposed to a high fibre ration. The relative proportions of microbe populations within the rumen are influenced by diet and have effects on productivity and systemic health of the cow. If the microbiome is poorly adapted to the diet, animal feed conversion will be decreased, and the animal will be a poorer performer. Digestive disorders such as acidosis can also occur, elevating circulating pro-inflammatory cytokines and increasing susceptibility to disease. With improvements in technology, it is now possible to better understand the modulation of gut microbiota in relation to feed conversion efficiency and the immune response. Characterising variations in the gut microbiome from farm of origin and throughout production

systems may lead to improvements in production performance and is fundamental to designing strategies to prevent disease and maintain healthy animals.

Index of Abbreviations

%WG: percentage weight gain

316v: MAP antigen (see Table 4.2.1)

ACC: Australian Country Choice

ACTB: actin beta

ADG: average daily gain

AEC: Animal Ethics committee

AFEC: Average Faecal egg count

ANOVA: Analysis of variance

BOLA-Ia: Bovine Major Histocompatibility Complex (BOLA) class I histocompatibility antigen, alpha chain BL3-7-like

Bovine Respiratory Disease (BRD)

C strain: Cattle strain (of *Mycobacterium avium* subsp. *Paratuberculosis*)

C10H15orf48: *Ovis aries* chromosome 7 C15orf48 homolog

C19orf12: Bos taurus C19orf12 homolog (LOC786987)

CAMs: Cell adhesion molecules

cDNA: complementary deoxyribonucleic acid

CFU: colony forming units

CHCHD1: coiled-coil-helix-coiled-coil-helix domain containing 1

CO_2: carbon dioxide

Con A: Concanavalin A (see Table 4.2.1)

COPG2: Coatomer protein complex subunit gamma 2

cRNA: complementary ribonucleic acid

CSF1: colony stimulating factor 1

Ct: Cycle threshold (of quantitative polymerase chain reaction)

CXCL_: C-X-C motif chemokine ligand (eg. CXCL10)

E: Efficiency (of quantitative polymerase chain reaction)

EBV: Estimated breeding values

ELISA: enzyme-linked immunosorbent assay

EMAI: Elizabeth MacArthur Agricultural Institute

FC: fold change (in gene expression)

FCM: faecal cortisol metabolite

FCN1: ficolin (collagen/fibrinogen domain containing) 1

FDR: false-discovery rate

FLA-ST: Flagellin (see Table 4.2.1)

FWEC: Faecal Worm Egg Count

GEO: Gene Expression Omnibus

GRO-1: melanoma growth stimulating activity alpha, also called chemokine (C-X-C motif) ligand 1/2

GZMB: Granzyme B

H3F3A: H3 histone, family 3A

HbF: hemoglobin fetal subunit beta

HcL3: *Haemonchus contortus* third stage larvae

HPA: hypothalamus-pituitary-adrenal

HSPB1: heat shock 27kDa protein 1

HT-J: high throughput Johne's direct PCR assay

ICAM1: intercellular Adhesion Molecule 1

IC-PMS: Inductively coupled plasma mass spectrometry

IFNα: Interferon alpha

IFNβ: Interferon beta

IFN-γ: interferon gamma

IL-: interleukin- (eg. IL-10)

IL-1α: Interleukin 1 alpha

IL-1β: Interleukin 1 beta

IMPA2: inositol(myo)-1(or 4)-monophosphatase 2

IP-10: Interferon-gamma inducible protein 10, (also termed CXCL10)

IPA: Ingenuity® Pathway Analysis

IRF7: Interferon regulatory factor 7

KEGG: Kyoto Encyclopedia of Genes and Genomes

KLRC1:

LPS: Lipopolysaccharide (see Table 4.2.1)

LXN: latexin

LYZ1: lysozyme 1

MAP: *Mycobacterium avium* subsp. *paratuberculosis*

MCP-1: Monocyte Chemoattractant Protein 1

MDC: Meat and Livestock Australia Donor Company

MIP1β: Monocyte Inflammatory Protein 1 beta

MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments)

mRNA: messenger ribonucleic acid

MSA: Meat Standards Australia

NCBI: National Centre for Biotechnology Information

NVLS – no visible lesions

OJD: ovine Johne's disease

Pam3CSK4: Pam3CysSerLys4 (see Table 4.2.1)

PAMP: Pathogen Associated Molecular Pattern

ParaTb: paratuberculosis

PBS: phosphate buffered saline

PCA: Principal Component Analysis

PM: perfect-match (probe distribution for microarray)

poly(I:C): Polyinosinic-polycytidylic acid (see Table 4.2.1)

ppb: parts per billion

PPDa: purified protein derivative from *M. avium*

PPIA: peptidylprolyl isomerase A

PTGR1: prostaglandin reductase 1

PWG: Percentage body weight gain from induction (into feedlot)

PWM: Pokeweed mitogen (see Table 4.2.1)

qPCR: quantitative polymerase chain reaction

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

RARRES1: retinoic acid receptor responder 1

REML: restricted maximum likelihood linear mixed model

RMA: Robust Multichip Averaging

RTL: relative telomere length

SAMRC: Southern Australian Meat Research Council

SNP: single nucleotide polymorphism

STAT1: Signal transducer and activator of transcription 1

TES: testin LIM domain protein

TET2: tet methylcytosine dioxygenase 2

Th: T helper lymphocyte subset

TLR: Toll-like Receptors

TMB: tetramethylbenzidine

TNFα: tumor necrosis factor alpha

TNFRSF21: TNF receptor superfamily member 21

Trace elements (from periodic table): sodium (Na), magnesium (Mg), aluminium (Al), silicone (Si), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), thallium (Ti), vanadium (V), chromium (Cr), magnesium (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), silver (Ag), cadmium (Cd), barium (Ba), lead (Pb).

Tslpr: thymic stromal lymphopoietin receptor

VEGF: vascular endothelial growth factor

wpcc: weeks post co-challenge

ZFP75A: zinc finger protein 75A-like

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1. Background

1.1 Introduction

This project aimed to provide an objective measure of an animal's immune fitness, otherwise termed immunocompetence. Immune fitness enables production animals to respond to challenges affecting health, well-being and productivity. Such a measure would assist industry stakeholders to evaluate the positive impact of changed management procedures and to support productivity ("fit for purpose").

In Australia, the cost of ill thrift due to stress and disease to livestock during the production cycle can range between $20-115 million annually, leading to reduced growth rates, condemned carcasses, downgrading of meat, wool and skin, and restriction of trade (Lane et al., 2015). Research has consistently linked animal husbandry associated stressors (Chen et al., 2015) with decreased immune function leading to direct impacts upon disease susceptibility and the ability of animals to thrive (Amadori & Zanotti, 2016), as shown in Fig.1.1. The capacity of an animal to mount an immune defence, or in other words, to display immunocompetence or immune fitness, has a genetic, nutritional, and environmental basis through epigenetic changes, with potential influence by the gut microbiome. Overall, it determines the ability of an individual to overcome pathogens (resistance) and cope with their effects (resilience).

Psychological stress
- Co-mingling
- New environments
- Unfamiliar/loud noise
- Restraint

Physiological stress
- Disease
- Nutrient deficiencies
- Endocrine disruptors
- Trace element deficiencies

Physical stress
- Hunger
- Thirst
- Fatigue
- Thermal
- Injury

- Altered heart rate
- Altered response to infection
- Increased fear response
- Increased susceptibility to disease
- Weight loss
- Behavioural changes
- Immune suppression
- Altered feed intake

- Decreased average daily weight gain
- Increased mortality
- Reduction in reproductive ability
- Decreased milk production
- Altered metabolic function
- Increased vocalisation, ambulation and aggressive behaviour

Figure 1.1: Three broad stressor categories applied to production animals. Psychological (such as new environments, exposure to human handlers and social hierarchy), physiological (such as nutrient or trace mineral deficiencies) and physical (such as heat stress and disease) stress can lead to changes in biological functions, which can result in the overall loss of welfare. Based on (Chen et al., 2015)

Livestock have long been bred to encourage the inheritance of production or disease resilience traits that result in high yield (Adams & Templeton, 1993), for example dairy cattle have been bred for enhanced resilience to brucellosis (Price et al., 1990) and mastitis (Heringstad et al., 2003) and *Bos taurus* beef cattle have historically been crossbred with cattle tick (Frisch, O'Neill & Kelly, 2000) and heat-stress resilient *Bos indicus* breeds. However, alongside breeding for specific disease resilience there is recognition that breeding for a specific trait may have potential impacts on the health or welfare of an animal and has sometimes been associated with compromised health outcomes. In extreme cases, enhanced susceptibility to other diseases has been found (van der Most et al., 2010). This leads to the requirement for a balanced approach that takes in the need to select for animals able to maintain a beneficial immune response during periods of high stress and achieve the desired

production outcomes. The University of Guelph in Canada has patented 'High Immune Response' genetics (Mallard et al., 2015) which classifies cattle based on Estimated Breeding Values for immune responses and has been shown to have a positive impact on the reduction of infections such as mastitis. These researchers acknowledge that immune responses should be "kept in balance when selecting for enhanced disease resistance", however what contributes to optimal immune balance in the face of livestock productivity under challenge remains largely unknown. An analysis of overall immune fitness has not been progressed in the livestock production area however Hine and others considered heritable immune phenotypes as a model for assessment of immune competence in their model of resilience (Hine, Cartwright & Mallard, 2012).

An improved understanding of the interrelationship of overall immune health of an animal under production stresses such as handling, feedlots, transportation or lairage and following exposure to disease catalysing pathogens, is essential to maintain health, high yield, and exemplary animal welfare. Insight to this aspect would potentially provide the tools for improved management and the development of therapeutics to adapt the immune response to a more favourable profile.

1.1.1   Immune factors

Immunocompetent animals fare better in the face of physiological challenges such as exposure to infectious diseases. Additionally, management-related stressors can impact on an animal's ability to thrive, as it is known that stress can suppress immune responses. Prior research has identified negative modulation of the immune response as a significant factor in both susceptibility to infection and an overall failure to thrive, resulting in reduced welfare and profitability. A measured response when exposed to environmental microbes or other psychological and physical stressors and a contained but potent response to pathogens could be the key to immune fitness. Potential on-farm and post-farm gate stressors or 'pressure points' include vaccination and drenching, transportation, feedlot and lairage.

Traditional measures of stress in animals and humans have focussed on the hypothalamus-pituitary-adrenal (HPA) axis (Fig. 1.2). Increased levels of blood glucocorticoids are often used as a key marker to characterise the stress response (Cockrem, 2013). However, blood sampling in itself is invasive and together with associated handling procedures can cause an increase in circulating glucocorticoid levels within five minutes (Touma & Palme, 2005). Faecal and hair cortisol measurements are alternative, non-invasive approaches to assess glucocorticoid levels (Gow et al., 2010; Narayan, Sawyer & Parisella, 2018). Measurement of faecal cortisol has been found to be accurate due to the ability to indicate blood cortisol levels from the HPA axis, reflecting the responses from several hours or even days prior to excretion (Hernandez-Cruz et al., 2016; Sheriff,

Krebs & Boonstra, 2010). Similarly, hair cortisol measurements are insensitive to the impact of acute stress, for example from handling, and the hair can accumulate glucocorticoid hormones over a period of weeks to months and is therefore a better estimate of longer term stress (Gow et al., 2010; Pereg et al., 2011). Both faecal and hair or wool cortisol measurements have been used in previous studies of sheep and cattle (Möstl et al., 2002; Mostl & Palme, 2002; Palme et al., 2000).
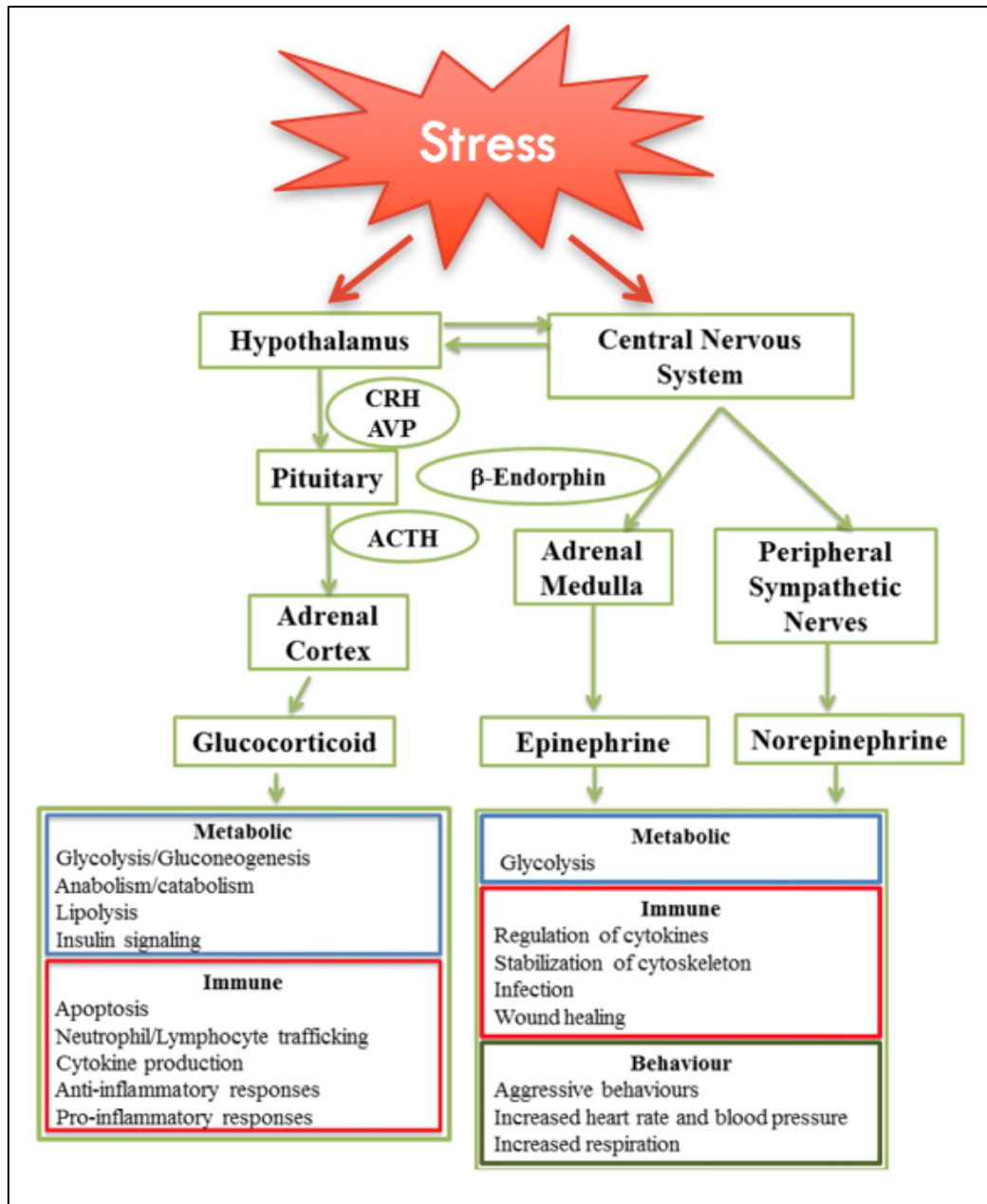
Figure 1.2: The effects of stress axes on biological functions. Hypothalamus-pituitary-adrenal (HPA) axis and the central nervous system are co-dependent and are both activated when stress is applied. With regards to immune fitness, the effect of stress varies. For instance, in the HPA pathway, it causes apoptosis while in the central nervous system, an individual is susceptible to infection. Based on (Chen et al., 2015)

Immune responses between individual animals can vary. Research undertaken by the Milieu

Interieur Consortium (Thomas et al., 2015) recognises that immune responses in humans are highly

variable dependent upon genetic predisposition, life experience, exposure to environmental stress and pathogens, and this presents challenges in terms of therapeutics and treatment. The Milieu Interieur project seeks to define factors that contribute to and characterise this inter-individual immune response heterogeneity. Early reports suggest an analysis of the transcriptome provides a route to discern gene and protein expression signatures of induced immune responses for correlates of protection (Duffy, 2018) and reports a strong link between host health and the gut microbiome since altered microbiota diversity was consistently linked to increased risk of several health outcomes (Partula et al., 2019).

When considering the overall immune fitness of an animal, their nutritional status is a key aspect. Optimal nutrition and adequate levels of trace minerals (elements) enable an animal to function optimally, with appropriate structural, physiological, catalytic and regulatory processes (Suttle, 2010). A number of trace minerals are essential for mammals and these play a range of important roles in biological processes, including involvement in oxygen transport, maintaining hormonal levels, and catalysing enzymatic reactions. Mineral requirements are difficult to establish, with most estimates in livestock based on the minimum level required to avoid symptoms of deficiency rather than levels required to promote optimal productivity. Key trace elements in cattle and sheep include copper (Cu), selenium (Se), zinc (Zn), manganese (Mn), iodine (I), and cobalt (Co) (Lee & Knowles, 2002; National Academy of Sciences, 2016). As part of the overall assessment of the immune fitness of animals included in this study, a serum trace mineral assessment was undertaken to exclude a particular mineral deficiency as being an explanatory factor in the associations found.

1.1.2   Genetic factors

Changes in the external and internal environment related to stress (as above Fig 1.1) can lead to many changes in the physiological responses of the animal, including growth rates, metabolism, and immune function. These physiological changes occur because of cellular changes that are measurable at the level of gene expression. Transcriptomics is the study of an organism's transcriptome, the sum of all its RNA. This is both the mRNA as well as the non-coding RNAs, which have a variety of functions. Essentially, gene expression shows us functional aspects of the genetic code written on our DNA. Transcriptomic studies allow researchers to determine when and where genes are turned on and off, enabling an understanding of an organism's response to changing conditions. This can be utilised to study an individual's response to stress.

High-throughput experimental techniques such as microarray, RNA-Seq and other next-generation sequencing techniques have been widely applied to monitor genome-wide DNA, RNA and epigenetic molecular activities and to detect disease-associated events or biomarkers (Bhattacharya & Mariani,

2009). The high experimental costs related not only to the generation of 'omics datasets but also the generation of primary samples with broad reproducibility often limit the number of samples analysed. This has led to queries regarding the statistical power and reproducibility of small sample studies and on occasion this concern has been justified in poor validation of putative biomarkers in real-world applications. The convention for the storage of 'omics datasets to public data depositories such as the Gene Expression Omnibus (GEO) from the National Centre for Biotechnology Information (NCBI) provides the opportunity to utilise a meta-analysis approach to utilise multiple independent yet related datasets for analysis. This is not a novel concept and has been reported in over 690 peer-reviewed publications in relation to human disease (Barth et al., 2006; de Magalhaes, Curado & Church, 2009; Goonesekere et al., 2018), agronomy (Sirohi et al., 2020) and physiological functions in livestock (Farhadian et al., 2018). The robust nature of the predicted signature regardless of variations in tissue source or experimental setup (Dudley et al., 2009; Glicksberg et al., 2019) makes this a stable model for prediction of genetic correlates of immune fitness. The meta-analysis of curated microarray datasets associated with livestock exposed to diseases or environmental stressors provides a useful tool for identifying correlates of immune fitness. This approach can be layered to encompass multiple variables including the identification of genes involved in a baseline immune response to all stressors as well as those genes associated to resilience to disease and other stressors.

It may be argued that an ideal estimate of an animal's true welfare status is a marker that measures cumulative experience, which can be defined as the sum total of all the positive and negative experiences in its life. The cumulative experience directly relates to the animals' biological age; animals with objectively negative experiences will have biological ages far beyond their chronological age due to stress. Telomere length could be a cumulative indicator of how an animal responds to stressors over time (Bateson, 2016). Telomeres are repetitive DNA sequences found at the ends of chromosomes. They function as 'caps' to prevent destruction of DNA during replication and repair. At each cell division, a few base pairs are lost and telomere length shortens. Once a cell reaches the maximum number of times it is programmed to divide, the cell enters replicative senescence and subsequently dies. There is an established link between stress and aging, with chronic stress leading to acceleration of aging, and therefore mortality (Eugene, Bourgeron & Xu, 2017). Constant exposure to stressors can increase an individual's risk for developing diseases such as cardiovascular, autoimmune and neurodegenerative disorders, and cancer (Wilbourn et al., 2018; Wolkowitz et al., 2011). Development of these diseases can be attributed to decreased function and senescence of cells *en masse* over time (i.e. aging) (Nettle et al., 2015), oxidative stress increasing the rate of cell turn over (Coluzzi et al., 2014; von Zglinicki, 2002), and the immune system

dampening telomeric DNA repair (Bateson, 2016; Plot et al., 2012; Shiels et al., 1999). An organism's chronological age and biological age are therefore not always identical. Human studies have shown that chronic stress can increase biological age by a decade, which would increase an individual's risk of mortality from all causes far beyond age-matched controls (Fairlie et al., 2016; O'Donovan et al., 2012; Shalev et al., 2013). Past studies have utilised relative telomere length (RTL) of blood leucocytes as an objective measure of welfare status and biological age (Bateson, 2016). This technology has recently been adapted and validated in livestock (Seeker et al 2016).

1.2 Benefits to producers

The innovative approach undertaken in this MDC project is not limited by previous dogma regarding optimal immune response and acknowledges that there will be more than one challenge (both infectious and non-infectious) to an animal's health and welfare throughout the production chain (Fig. 1.3). A tool that provides an objective measure of immune fitness or immunocompetence can be applied to examine multiple production challenges.
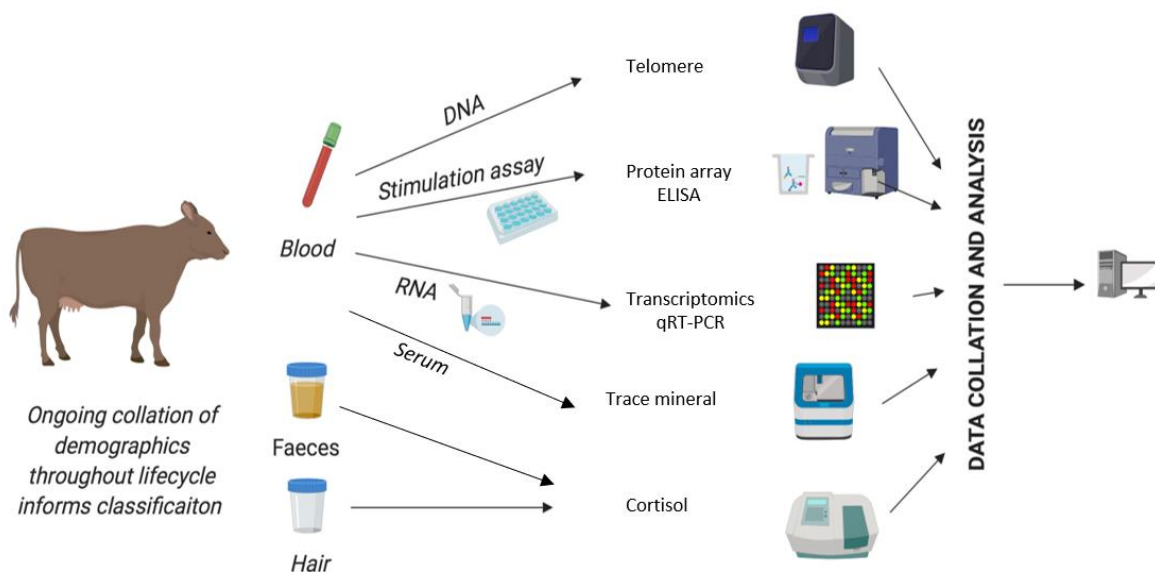
Figure 1.3: Experimental model for dissecting immune response variability in sheep and cattle. Biological samples sourced from sheep and cattle exposed to pathogens and stressors relevant to the Australian red meat industry were analysed by a wide range of techniques including Telomere analysis ('biological age'), Proteomics (protein arrays and ELISAs to detect proteins produced in response to immune stimulants), Transcriptomics and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for gene expression (i.e. which genes are switched 'on' or 'off'), serum trace mineral assessment and determination of stress markers (cortisol), to identify putative biomarkers of immune fitness.

The benefit of knowing the immune fitness status of an animal can be readily envisaged. Production stresses encountered by young animals during transport, prior or during finishing in feedlots, are

already being addressed in other countries. ZelNate® an 'immunostimulant product' is marketed overseas to combat the impact of transport stress on young animals (Shankar, 2016). It is a bacterial plasmid DNA liposome-based immunomodulator that claims to stimulate the innate immune response to enhance protection of cattle against *Mannheimia haemolytica*, one of the causative agents of Bovine Respiratory Disease (BRD) (Ilg, 2017). Improved understanding of the overall immune fitness of an animal will enable a producer to make a judicious decision on a cohort of animal's suitability to the production enterprise; for example, whether to send them to feedlot finishing. Indications of the flock/herd's overall immune fitness might provide justification for adaptation of enterprise or animal husbandry procedures to improve animal wellbeing.

This program directly addressed key points in the production process which can impact animal welfare; a priority area for the red-meat industry that has recognised that poor understanding of animal welfare and inappropriate measures to address current practices in production animals represent an enormous risk to the industry, which is estimated to grow to more than $3 billion by 2030 (Meat Industry Strategic Plan 2020). The Southern Australian Meat Research Council (SAMRC) in their Research, Development & Adoption Plan 2016 also describes animal health, welfare, and productivity as being inextricably linked. There is a need to identify, analyse and address those events which impact on the welfare, productivity, and potential of an animal during its life.

We aimed to develop simple immune measure(s) as a correlate of physiological health and well-being for use as a benchmarking tool for overall herd health and welfare. This immune fitness toolkit and biomarkers of Immune fitness identified in sheep and cattle could be applied to assist producers in identifying improvements to welfare and selective breeding.

2. Objectives

1. A prototype score card to categorise animal immune fitness comprising of:
    i. A suite of immunological tests for identifying immune factors that may affect the welfare and health of animals, which are impacted by production processes
    ii. Genetic correlates of infection
2. Identification of critical pressure points impacting immune fitness along the production chain and thus identification of areas of risk to welfare and productivity which will enable integration of welfare through the value chain.

3. Identify and validate a baseline for immune fitness linked to red meat quality. Assess parameters in association with health, disease, and production outcomes.

3. Methodology

3.1 Animal models

3.1.1 *Mycobacterium avium* subspecies *paratuberculosis* and *Haemonchus contortus* co-challenge trial

Endemic infections cause significant welfare impacts for animals and are a major stressor in livestock productions systems, as well as leading to significant production losses. Two diseases of relevance to sheep production in the Australian setting are ovine Johne's disease (OJD), caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and intestinal parasitic infections, such as *Haemonchus contortus* (Barber's pole worm) (Emery, Hunt & Le Jambre, 2016; Whittington et al., 2012)

A long-term (1 year) exposure trial conducted within an aligned MLA Donor Company project (P.PSH.0813; Resilience on-farm: Mechanisms, Markers and Applications) provided samples for identification of immune markers of disease resilient/susceptibility. Aspects of this trial outcome have been reported in the final report of project P.PSH.0813.

With approval from the University of Sydney animal ethics committee (AEC Protocol No. 2017/1249), 90 weaner Merino and Merino-cross lambs from an OJD MN3 status flock at Armidale were exposed to MAP (Telford 9.2) alone or in combination with 800 *Haemonchus contortus* third stage larvae (*H. contortus* HcL3; Kirby Strain). Sheep were drenched on arrival and were all Faecal Worm Egg Count (FWEC) negative prior to exposure. Animals were run together (except 10 uninfected as a control group) and progressively rotated through 7 paddocks at 4-weekly intervals to keep ahead of the life cycle of *H. contortus*, and minimise the inadvertent acquisition of an additional worm challenge.

The groups for the co-challenge study are shown in Table 3.1.1. The HcL3 were administered orally in 10 ml water containing 200 HcL3 at weekly intervals for 4 weeks; with the first dose a week prior to the first MAP dose. MAP Telford 9.2 was administered in 10 ml PBS orally. Three doses were given on weeks 1, 2 and 5 with each dose containing (1.13×10^9 doses 1&2 and 7.70×10^8 dose 3) live MAP. Groups 5 and 6 served as sentinels for parasitic pasture burden and were subjected to two different drenching regimes. Group 7 was maintained on a separate paddock as a paratuberculosis-free control group for comparison of exposed and unexposed individuals.

Table 3.1.1: Groups in the MAP and *H.contortus* co-challenge trial.

Group	Number	Treatment
1	20	*H.contortus* + MAP (normal drenching[*])
2	15	MAP (normal drenching[†])
3	15	MAP (suppressive drenching)
4	20	*H.contortus* (normal drenching[*])
5	10	*H.contortus* controls (normal drenching[†])
6	10	*H.contortus* controls (suppressive drenching)
7	10	MAP controls (uninfected)(suppressive drenching)

Normal drenching: * when Faecal Worm Egg Count (FWEC) exceeded 5000 eggs per gram (epg) of faeces in 2 successive counts for *H.contortus* challenged groups; † when FWECs exceeded 500 epg on 2 successive counts for non-challenged groups. Suppressive drenching was carried out prior to animals being moved to a new paddock, every 4 weeks.

Blood, faecal and wool samples were collected from all animals prior to exposure and at regular intervals thereafter. Sheep were weighed periodically throughout the trial to determine average daily gain (ADG) and percentage weight gain over the duration of the trial (%WG). ADG is a marker of disease progression for paratuberculosis, with rapid weight loss an indicator of the onset of clinical disease. Percentage weight gain over the duration of the trial was selected as a measure of productivity.

The animals were sacrificed upon evidence of clinical paratuberculosis i.e. ≥10% weight loss within a month or at the conclusion of the trial. Upon necropsy faecal, blood and tissue samples were harvested for faecal and tissue culture, high throughput direct PCR assay (HT-J) (Plain et al., 2014; Plain et al., 2015) and histopathology to verify clinical disease and accurately classify disease outcome. A paratuberculosis disease severity score (0-100) was calculated based on cumulative evidence from tissue culture, histopathological lesion grade and evidence of gross lesions (reported in **Appendix 8.5**).

Faecal egg counts were performed for the duration of the trial at 2-weekly intervals to monitor the establishment and progress of the helminth infestation. A drenching decision of >5000 eggs per gram (epg) on successive FWECs ("normal drenching") was established to avoid clinical disease and prevent over-contamination of paddocks for treatment groups experimentally inoculated with *H. contortus* (Groups 1 & 4), for treatment groups not experimentally inoculated with *H. contortus* and maintained under a 'normal drenching' regime where drenching was administered at >500 epg (Groups 2 & 5). To optimise performance and minimise parasite effects, "suppressive" drenching was performed on some cohorts (Groups 3, 6 & 7) prior to all animals being moved to a new paddock at 4-weekly intervals. Initially all animals were drenched and then drench type was rotated every 6 months based on FWEC. Average FEC was calculated across all timepoints assessed

commencing from one-month post-HcL3 inoculation until the time of first drenching, or until the end of trial if the animal did not need to be drenched.

3.1.2   *Salmonella typhimurium* sheep challenge model

This short-term (50 day) infection trial provided samples for identification of immune markers of disease, insight of pressure point variation (pre and post grazing nutrient source and exposure to environmental stress consistent with sheep transitioning to export).

The *Salmonella typhimurium* challenge model in sheep was conducted according to a previously published model (Mohler et al., 2012). Briefly, 15 Merino or Merino-cross wethers were sourced from a commercial property and transported to the Elizabeth MacArthur Agricultural Institute (EMAI) where they were acclimatised to an intermediate animal housing facility (holding pens) in small groups (~4 sheep/group). The sheep were approximately 6 months of age at purchase and had been acclimatised to a pasture grazing based nutrition source. Upon arrival at EMAI, the sheep were transitioned to a feed profile that included commercial oaten chaff and pellets, consistent with the export industry. Sheep entered segregated animal housing 7 days prior to challenge with a single oral dose at 10 mL oral bolus re-suspended in phosphate buffered saline (PBS) of *Salmonella typhimurium* at 1×10^9 colony forming units (CFU). Within the segregated housing, sheep were individually housed in wire mesh pens on raised wire flooring ensuring separation from urine and faecal material. Pens were cleaned daily. Each pen contained buckets for oaten chaff, pellets, and water. All sheep were sacrificed 30 days post exposure to Salmonella unless they displayed criteria requiring unscheduled euthanasia.

Faecal and blood samples utilised for immune measure analysis were collected, and weight was measured from individual animals on farm (day -28), following transport to EMAI, segregation to small groups and transition to pellets/chaff (day -7) and 7 days post exposure to *Salmonella typhimurium*. Additional samples were collected for the purpose of infection monitoring, briefly faecal samples were assessed for *Salmonella* utilising an enrichment culture to provide a positive or negative status and quantification of bacterial load was determined by quantitative (q)PCR.

3.1.3   Commercial property sourced sheep (Arthursleigh)

Merino and merino-cross sheep samples sourced from Arthursleigh, a commercial property located in Big Hill, NSW, over a period 7 months (210 days) provided a source for identification of baseline immune fitness measures and verification of predicted markers. Due to the 2019-2020 bushfires, severe flooding in February of 2020 and Coronavirus pandemic-mandated travel restrictions, the sampling model was adapted.

With AEC approval number (Protocol No. 2017/1234), 220 Merino and Merino-cross lambs were randomly selected from the flock on Arthursleigh. The lambs were run in a flock of approximately 1400 sheep that included 500 lambs. At the age of 4 months, each randomly selected lamb was marked with a secondary tag to enable re-sampling. The sheep were maintained on the commercial property under standard Australian management conditions.

Blood, faeces, and wool samples were collected, and weight was measured from all 220 animals at 4 months and again at 11 months of age. Production outcome was determined by percentage weight gain and average daily gain between the two sampling events.

3.1.4 *Mycobacterium avium* subspecies *paratuberculosis* sheep exposure trial (P.PSH 0311)

A previous long-term (2.5 years) trial provided Affymetrix GeneChip™ array-derived data for identification of genetic correlates of immunity. The final report for this MDC funded project (P.PSH. 0311) has been published and the transcriptomic research is published (Purdie et al., 2019b).

3.1.5 Mycobacterium avium. subspecies paratuberculosis exposure trial in vaccinated sheep (P.PSH.0576)

A previous trial provided Affymetrix GeneChip™ array-derived data for identification of genetic correlates of immunity in vaccinated sheep. The final report for this MDC funded project has been published (P.PSH.0576) and further validation of genes associated with resilience and susceptibility to Johne's Disease in commercial properties was reported in the final report for P.PSH.0813 (submitted 31st March 2021).

3.1.6 Brisbane Valley ACC feedlot 78 day short-finished cattle

With AEC approval number (Protocol no.2017/1234) blood, tail hair and faecal samples were sourced from 49 cattle undergoing finishing at the Australian Country Choice (ACC) operated Brisbane Valley Feedlot. The sampled cattle were randomly selected at induction from a cohort of 80 mixed breed cattle (*Bos taurus* (n=33) and *Bos taurus* X *Bos indicus* (n=47)) obtained by ACC from four affiliated producers located within the Scenic Rim Region of South East Queensland. The farms contributed a mix of breeds and sexes to the feedlot for a 78-day short finishing-term prior to slaughter. All 80 cattle were inducted to the feedlot on the same day and were housed together thereafter. Following induction the cattle were transitioned from a pasture grazing diet to pelleted ration in a 3-stage process (induction, transition, full ration). The sampled cohort (n=49) consisted of eleven breeds (Brahman, Angus, Brangus, Santa Gertrudis, Droughtmaster, Charbray, Braford,

Murray Gray, Red Angus, Hereford and Charolais) with a ratio of n= 31 *Bos indicus* x *Bos taurus* to n=18 *Bos taurus*.

Repeat sampling of the 49 randomly selected cattle took place at induction (day 0), post acclimatisation to feed transition (day 42) and prior to slaughter (day 72). All health and welfare measures including weights were collected (days 0, 42, 72 and 78) and recorded and any animals identified as unhealthy through the daily monitoring by handlers (e.g. cessation of feeding, voluntary separation from the herd, appearance of malaise) were separated from the cohort and treated as per the Animal Ethics requirements. At slaughter hot carcase weight, MSA grading outputs and general health observations were recorded. These data were utilised to assign variables for ongoing analysis. Percentage body weight gain from induction (PWG) and ADG were calculated and utilised to identify cattle with the highest performance metrics and lowest performance, those least fit within the acceptable range (≤ 400 Kg, ≤ 25% PWG or ≤ 1.2 kg ADG). Animals gaining ≥ 30% PWG and ≥ 1.6kg per day (ADG) were classed as high weight gain achievers. The remaining cattle were classed as mid-range achievers.

3.1.7 *Mycobacterium avium* sub-species *paratuberculosis* experimental infection trial (P.PSH.0297)

A previous long-term (4.5 year) infection trial provided Affymetrix GeneChip™ array derived data for identification of genetic correlates of immunity and biological samples for validation. The final report for this MLA funded project has been accepted and published (P.PSH 0297) and this work has been published in a peer reviews journal (Purdie et al., 2012). Briefly, A controlled experimental model of bovine Johne's disease was established utilising a previously validated sheep infection model (Begg et al., 2010). Twenty Holstein and Holstein Red calves aged between 2 to 4 months were inoculated three times over the period of a month with a low passage laboratory seed stock culture of MAP cattle (C) strain CM00/416. The cumulative dosage of the challenge was 4.73 x 10^8 viable MAP cells. Blood samples were collected from the 20 exposed and 10 unexposed control animals prior to inoculation and at monthly intervals thereafter. Regular faecal samples were collected for culture analysis and progression of clinical signs was closely monitored. The cattle were sacrificed four and a half years post primary exposure to MAP.

3.1.8 Novel vaccine trial (Camden, NSW)

Samples were collected from a short-term (5 months) trial undertaken to develop a novel vaccine under approval (AEC Protocol No. 2019/1582). Thirty Angus steers were purchased from a commercial saleyard and transported to Camden. Animals were allocated to three groups (n=10 per

group) after acclimatisation to the experimental site; this was based on initial weight to ensure that the variation in weight was similar between groups. All animals were orally dosed at the start of the trial. The control group received sham vaccine while the other two groups received the prototype vaccine. One of the vaccine groups received two more doses one week apart. At the time of the first dose animals were introduced to grain. Access to pasture and grain feed was maintained for 6 weeks after which the cattle only had access to pasture. Tail hair samples were collected for cortisol analysis and animals were weighed regularly. The trial was terminated at 5 months after the first dose.

3.1.9 **Bushfire exposed cattle**

Samples were collected from cattle that were on a property in NSW that was fire affected in the 2019-2020 bushfire season, as well as well as from a control, unaffected property. The cattle were on-farm during the time the fires went through the affected property. This was thought to be a unique type of stressor that may be apparent in terms of long-term cortisol levels. Tail hair samples were collected from 12 animals on each farm as part of an MLA-funded project (B.AHE.2102: Health, welfare and biosecurity of livestock exposed to Australian bushfires). The project was covered by AEC Approval No. 2020-1777. These samples were utilised to assess hair cortisol levels.

3.2 Identification of immune factors

3.2.1 **Protein array**

3.2.1.1 Whole blood stimulation assay

Samples were sourced from sheep and cattle trials (3.1.1, 3.1.2, 3.1.3, 3.1.6 and 3.1.8). Blood was collected from the jugular vein of the sheep or the cattle into lithium heparin vacutainers. Equal volumes of blood and culture medium with or without stimuli were incubated for 48 hrs. Culture supernatants were stored at -20°C until cytokines were detected by protein array or ELISA**.**

3.2.1.2 Ovine and bovine cytokine arrays

An ovine array capable of detecting 20 cytokines (Raybiotech QAO) and a 30 cytokine bovine array (Raybiotech QAB Cyt) were used. These arrays follow a multiplexed sandwich ELISA-based quantitative array format which enables accurate determination of the concentration of multiple cytokines simultaneously (**Appendix 8.1**). Arrays were processed following manufacturer's instructions. The fluorescence intensity of each cytokine dot was scanned, normalised and analysed

in relation to the fluorescent intensity of recombinant cytokine standard curves using Q-analyzer software (RayBiotech).

3.2.1.3   ELISA validation of protein array

ELISA kits for ovine and bovine cytokines (Kingfisher Biotech) were optimised and used following manufacturer's guidelines with horse radish peroxidase-streptavidin and 3,3',5,5'-tetramethylbenzidine (TMB) for detection. Colour development was stopped with 2M H_2SO_4 and absorbance read at 450 nm. Standard curves using the relevant recombinant cytokine were used to convert optical density values to cytokine concentration.

3.2.2 **Measurement of Cortisol levels**

3.2.2.1   Faecal cortisol

Faecal samples for faecal cortisol metabolite (FCM) assessment were collected in tubes and flash frozen in dry ice to preserve cortisol integrity until arrival at the laboratory, where they were then stored in -80ºC.

Faecal samples were processed and extracted according to Brown and Hernandez-Cruz (Brown et al., 2001; Hernandez-Cruz et al., 2016) with some modifications. Frozen samples were incubated for two hours at 97ºC in a laboratory incubator. After two hours, the samples were removed and individually mashed with paddle pop sticks into a smooth consistency. Then, 0.5 g of faecal material from each sample was added to 5 ml of 80% methanol and placed on a shaker for 30 min for extraction of cortisol metabolites. The samples were then centrifuged at 2500 x g for 15 min, after which the supernatant containing cortisol metabolites was recovered and stored in a cryogenic vial at -80ºC until analysis. FCM were measured using a competitive Cortisol enzyme-linked immunosorbent assay (ELISA) kit from Cayman Chemicals. The detection range of the assay was 6.6 to 4000 pg/ml and the ELISA has a sensitivity (80% B/B0) of approximately 20 pg/ml.

Full details of the methods are provided in **Appendix 8.2**.

3.2.2.2   Hair (wool and tail hair) cortisol

Sheep wool samples and cattle tail hair samples were stored protected from light. A wool sample was submitted for each animal in the study. For cattle tail hair, the hair was cut into four segments, each 3 cm in length. Segment 'a' was the first 3 cm segment of tail hair closest to the animal, whereas segment 'd' was furthest away. Hair/wool cortisol measurements were performed by

Stratech Scientific (https://www.stratechscientific.com.au/services/hair-analysis/). Results were provided as Cortisol level (pg/sample) for cattle or cortisol levels (pg/100mg) for wool.

3.2.3   Trace mineral analysis of serum

Serum trace minerals were assessed using a validated protocol for cattle serum (Luna et al., 2019). This involved a single acid digestion of the serum prior to Inductively coupled plasma mass spectrometry (IC-PMS). The serum (300 µl) was aliquoted into 2 ml eppendorf tubes. Within a fume hood, 300 µl concentrated nitric acid and 150 µl hydrogen peroxide was added to digest the sample. The tubes were transferred to a heating block and incubated at 60°C for 2 hr in the fume hood with the lids open. After 10 min of this incubation, the tube lids were closed and they were briefly vortexed and pulse spun, then returned to the heating block with the lids open. After the incubation, the lids were closed, and the tubes were vortexed and centrifuged at 2000 rpm for 5 min. A 1 in 10 dilution was performed into labelled autosampler tubes for the IC-PMS and analysed. The standard used included the following elements: sodium (Na), magnesium (Mg), aluminium (Al), silicone (Si), phosphorus (P), sulphur (S), potassium (K), calcium (Ca), thallium (Ti), vanadium (V), chromium (Cr), magnesium (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), silver (Ag), cadmium (Cd), barium (Ba), lead (Pb). Results are reported as parts per billion (ppb) in the original sample.

3.3 Genetic correlates of Immunity

At the outset of the project a thorough analysis was carried out of publicly available gene expression datasets related to relevant research studies carried out on sheep, goat or cattle immune fitness including infectious and other welfare associated illnesses applicable to the Australian Red Meat Industry (MLA project report B.AHE. 0010) such as bovine respiratory disease, Johne's disease, parasite burdens, pestivirus (bovine viral diarrhoea), bovine ephemeral fever, tick fever and theileriosis. Initial analysis identified a knowledge gap in relation to gene expression datasets analysing cattle held under Australian feedlot conditions. This prompted transcriptomic analysis of gene expression from samples collected from cattle finished under feedlot conditions (section 3.1.6). Data derived from this study were analysed in part, by a postgraduate student as Doctor of Veterinary Medicine Research Project (**Appendix 8.3**).

3.3.1   Transcriptomic analysis of 78-day short finished feedlot cattle

The RNA samples of 12 cattle were selected from the cohort based upon low (≤ 25%) or high (≥30%) percentage weight gain over the period of the feedlot process and transcriptomic analysis was performed on samples collected from the same animals at induction (day 0) and prior to slaughter (day 72) (Table 3.3.1). A total of 24 individual samples were processed to GeneChips™. The remaining RNA samples were stored for qPCR analysis.

Table 3.3.1. Feedlot cattle selected for transcriptomic analysis based on the production measure percentage weight gain since induction.

Farm ID	Sex	Bos breed identity	ADG (kg)	% weight change	MSA Index
2	M	Bos Indicus x Bos Taurus	0.76	15	61
1	F	Bos Taurus	0.78	15	55
1	F	Bos Indicus x Bos Taurus	0.79	18	60
3	M	Bos Indicus x Bos Taurus	0.85	17	56
1	F	Bos Indicus x Bos Taurus	0.94	20	57
2	M	Bos Indicus x Bos Taurus	0.97	20	60
3	M	Bos Taurus	1.65	34	59
2	F	Bos Indicus x Bos Taurus	1.68	38	58
3	M	Bos Indicus x Bos Taurus	1.73	38	58
1	F	Bos Indicus x Bos Taurus	1.74	36	60
3	M	Bos Taurus	1.76	39	54
3	M	Bos Indicus x Bos Taurus	2.05	41	57

ADG: Average daily gain. % weight change: weight change proportional to initial weight at entry to the feedlot.

Blood samples were collected from the tail vein into PAXgene® Blood RNA tubes to stabilise RNA. RNA extraction was performed using the PAXgene® Blood RNA kit, according to manufacturer's instructions. Quantity and integrity of the isolated RNA was verified on a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific) and Agilent 2001 Bioanalyser analysis (acceptable RIN number 6.0-10). If required, an ethanol precipitation was performed to further purify the RNA. Samples were stored frozen at -80°C prior to use. The Affymetrix GeneChip™ Bovine Gene1.1 ST Array Strip, comprised of 526,801 probes translating to 24,341 genes and based on the UMD3.0 genome build was used for gene expression analysis. Briefly, 1µg of total RNA from each sample was reverse transcribed using a 3' IVT Express protocol as recommended by Affymetrix and hybridised to the array matrix.

The full details of the protocol of transcriptomic analysis of feedlot finished cattle are included in **Appendix 8.3.**

3.3.2 Meta- analysis of differential gene expression

Datasets were sourced from the National Centre for Biotechnology Information's Gene Expression Omnibus (NCBI GEO, https://www.ncbi.nlm.nih.gov/geoprofiles/advanced). The keyword searches ((("expression profiling by array"[DataSet Type] AND "bovine"[Organism]) OR "bos taurus"[Organism]) OR "Ovid Aries"[Organism])AND "disease" [All Fields] OR "stress" [All Fields]).

The full details of the protocols for sheep and cattle meta-analysis are included in **Appendix 8.3.**

3.3.3 Real time quantitative PCR verification of predicted correlates of immunity

Quantitative real time PCR (qRT-PCR) analysis was carried out on selected differentially regulated genes to verify the array findings and to assess genes of interest as correlates of immunity. Samples were selected from each of the sheep (animal model section 3.1.1, 3.1.2 and 3.1.3 and 3.1.4) and cattle (animal model section 3.1.6 and 3.1.7).

Samples were processed for qRT-PCR analysis. Briefly, species appropriate forward and reverse primer pairs were designed for selected gene regions of interest (Table 3.3.2) using the online software Primer 3 (Rozen & Skaletsky, 2000). The design of the primers ensured no amplification of genomic DNA contaminants.

Table 3.3.2. Primer sequences for putative correlates of immune fitness

Species specificity	Gene ID	Accession code	Primer sequence (5' to 3')	Source
Ovine	LYZ1	AH008120.2	GATGGCAAAACCCCTAACGC AGGGAGCAACCCTCCACATA	Designed
	RARRES1	XM_004003212.4	GCAGTGTCAAGCAGTGGAAAA ACCAAGTGAATACGGCAGGG	Designed
	TNFRSF21	XM_012100789.3	GATCGTGCTTTTCCTGCTGC TTCAGGATGTCAATACCGTGGC	Designed
	C10H15orf48	XM_004010648.4	TCGAAAAACCGATGTGATCCTT AGAAAAAGGCGAGGACTGGT	Designed
	BOLA-Ia	NM_001038518.2	TCACCCTGAGATGGGAACCT TCCTCCAGATCACAGCTCCA	Designed
	HbF	XM_004016241.3	GAGAACTTCAGGCTCCTGGG ATTGGCAACACCAGTCACCA	Designed
	LXN	XM_004003214.4	AGTTCGACATTTAGCCTGGGT AGTCATCATTTCTTTGCACTTGCT	Designed
	TET2	XM_027970835.1	GCCACACCCCAGCTTTAGA TACCCTTCTGTCCAAACCTTTCT	Designed
	TES	NM_001195318.1	GGCAGAAAACCAAAACTGGCA ATCCTTGACACACCACAGCG	Designed
	IP-10	NM_001009191.1	CCCACGTGTCGAGATTATTGCC AGCTGATTTGGTGACTGGCTT	Designed
	PPIA	XM_027968358.1	TGACTTCACACGCCATAAT	

Bovine	**ACTB**	NM_001009784	CTTGCCATCCAACCACTC TGGGCATGGAATCCTG	
	H3F3A	XM_004013633	GGCGCGATGATCTTGAT GAGGTCTCTATACCATGGCTC	
	TNFalpha	NM_001024860.1	GTACCAGGCCTGTAACGATG CCAGAGGGAAGAGCAGTCC	
	IL-1a	NM_001009808.1	GGCTACAACGTGGGCTACC GAACGACGCCCTCAATCAAAG	Designed
	KLRC1	NM_001319883.1	TTCACTGCCTCCTCCAGATTATTT ACAAGGCTCCAGAAAGAATGTCA	Designed
	IL-10	NM_174088.1	CAGCACAGTTATGTTCATCAGGT CTTTAAGGGTTACCTGGGTTGC	Designed
	GRO-1	NM_175700.2	CTCACTCATGGCTTTGTAGACAC GCTCCTGCTCCTGGTGG	Designed
	IMPA2	NM_001192282.1	TCAGTTGGCACTAGCCTTGTT TCTCAACCAAGACGTCAGCC	Designed
	HSPB1	NM_001025569.1	CTCCTCGGCAATGAACCTGT CACTCGCAAATACACGCTGC	Designed
	PTGR1	NM_001035281.2	GAGGATGKYCAGTGATGGCT GCCTGTTGGACATCTGTGGA	Designed
	CHCHD1	NM_001046484.2	CGGGAGCAGCTWCTTTCAGA CGACGCCAAGCCTCCG	Designed
	C19orf12	XM_024984039.1	AACTCCCCAACTCTCCCAGA GATAAACACAGCAGGTGGGC	Designed
			ATGGACAGTCACCTGGAGGA	

Three non-changing genes were selected as reference markers for each species; this follows MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Vandesompele et al., 2002). Selected RNA samples were reverse transcribed to cDNA using oligo(dt) primers and SensiFAST cDNA synthesis kit (Bioline), according to the manufacturer's instructions, with the addition of 1 µl RNasin® RNase inhibitor (Promega) per reaction to protect the RNA from degradation. qRT-PCR was performed using an Mx3000P Real-time PCR system (Stratagene, Agilent) using the QuantiTect SYBR Green PCR kit (Qiagen). Assays were prepared in 96 well plates and included duplicates of each sample. Reaction volumes of 25µl (including 10µl of target cDNA at a 1/100 dilution) were prepared and amplified under the following conditions: 95°C for 15min, then 40 cycles of 95°C for 20s, 52-60°C for 30s and 72°C for 30s, with fluorescence acquisition at the end of each annealing step. The specificity of the reaction was confirmed using melting curve analysis and standard curves were performed on each plate for each primer set.

3.3.4   **Assessment of telomere length following exposure to production stress**

Assessment of relative telomere length was performed using a modification of the protocol previously validated for sheep and cattle (Seeker et al., 2016). Briefly, DNA was extracted from whole blood using the Gentra Puregene Blood kit (Qiagen), according to the manufacturer's protocol with minor modifications. Quality control for the DNA samples was critical for performance of this assay (Seeker et al., 2016). DNA purity was assessed using a NanoDrop ND-1000 spectrophotometer with NanoDrop 2000 software. For DNA samples to pass quality control, they were required to have a DNA concentration minimum of 20ng/μl, an average 260nm/280nm absorbance ratio of above 1.7 and an average 260nm/230nm absorbance ratio of above 1.8 (indicative of low levels of contaminants e.g. salt, proteins). DNA quantity was then measured on a Qubit® 2.0 using a Qubit dsDNA BR Assay kit according to manufacturer's protocol. Samples were then prepared as 10ng/μl DNA working stock solutions and stored at -80℃ for quantitative PCR.

Relative telomere length (RTL) was measured by qPCR as the amount of telomeric DNA in a sample relative to the amount of a non-variable (single copy number) reference gene (Cawthon, 2002). For both sheep and cattle, the single copy number gene assay required optimisation and new primers were designed and used specifically for this purpose. In order to identify the most appropriate reference gene, a preliminary analysis we conducted examined a number of reference gene primer pairs. For sheep, the optimal primer set for the alternative sheep reference gene was H7.7, targeting the Coatomer protein complex subunit gamma 2 (COPG2) gene) (Hertz-Fowler et al., 2008). For cattle, a bovine primer set for Interleukin 1-alpha (IL-1α) was selected as the optimal primer set to use as the single copy reference gene for RTL measurements. Both the new sheep and the cattle reference gene primers were designed to recognise DNA within a single exon of the respective genes (non-intron spanning).

A quantitative (q)PCR assay was performed that targeted the Telomere and the reference gene relevant for each species. The genomic DNA was diluted from 10ng/μl to a concentration of 0.25ng/μl, of which 4μl was pipetted into each reaction to give a final DNA concentration of 1ng/μl. Each reaction contained 10 μl of Quantitect SYBR mix (Qiagen), the optimal concentration of the forward and reverse primer, 4 μl of template DNA, and nuclease free water to make a total volume of 20 μl per reaction, with cycling and data collection performed using an MX3000p qPCR instrument (Agilent). Each sample was tested in duplicate in the qPCR for both primers (Telomere and Reference gene) on identical quantitative PCR plates.

RTL was measured from the Ct values obtained for each sample using primers for telomere amplification and the optimally performing reference gene, according to the formula below.

Reactions with the tel1b/2b and IL-1α primers were performed in duplicate. The Ct value of the Calibrator sample and efficiency of the reaction was calculated for each experimental plate. The RTL for each sample was determined from the average of the duplicate reactions, using the following formula:

$$RTL = \frac{E_{TEL}^{Ct_{TEL(Calibrator)} - Ct_{TEL(Sample)}}}{E_{Ref}^{Ct_{Ref(Calibrator)} - Ct_{Ref(Sample)}}}$$

The full details of the protocols for sheep and cattle telomere measurement are included in **Appendix 8.5 and 8.6.**

3.4 Statistical analysis

3.4.1 Protein array

and

3.4.2 ELISA

Two-way ANOVA with multiple comparisons (Graphpad Prism 8.4.2) were conducted to determine significant differences (p<0.05) between stimuli and outcome groups (disease or weight).

3.4.3 Cortisol

Comparisons between faecal cortisol levels in the different treatment groups and time points in the sheep trial were analysed using a restricted maximum likelihood linear mixed model (REML), with faecal cortisol metabolites (FCM) as the outcome variable (GenStat 18th Edition, VSN International Ltd.). A random term of animal number was included in all models to account for individual animal trends. Differences between treatments, sampling periods and/or groups were evaluated using the standard error of difference. Wool cortisol measurements for the control versus exposed group of sheep were compared using a Welch's t-test. Significance was set at p < 0.05. Correlation between the various outcomes (FCM, wool cortisol, AFEC, HT-J DNA quantity and paratuberculosis score) were assessed either using a Pearson's correlation for parametric data or a Spearman's correlation for non-parametric data using GraphPad Prism (version 8.1.2). Hair cortisol measurements in different segments of hair for cattle were analysed using an ANOVA in GraphPad Prism (version 8.1.2).

3.4.4   Trace minerals

Correlation between the various outcomes were assessed either using a Pearson's correlation for parametric data or a Spearman's correlation for non-parametric data using GraphPad Prism (version 8.1.2).

3.4.5   Genetic correlates of infection

3.4.5.1   Transcriptomic dataset normalisation and baseline analysis

3.4.5.2   Affymetrix GeneChip™ operating software (GCOS) derived raw expression values were obtained as .CEL files and transferred to Partek Genomic Suite 7.1 software (Partek Inc) where all sample cohorts were analysed separately. Briefly, samples sets were tested to determine characteristics of high quality cRNA (3'/5' ratio of probe sets for the integral housekeeping genes of 1.5). The raw data were normalised using the RMA (Robust Multichip Averaging) algorithm (Irizarry et al., 2003). This method retains probe-level information and applies probe-specific background correction to compensate for any non-specific binding found in the chips using perfect-match (PM) probe distribution. In addition, RMA normalisation applied PM distributions across all the chips and a robust probe-set summary of the log 2 normalised probe-level data by median polishing. Array data quality was confirmed by Principal Component Analysis (PCA), which examined correlations between the data derived from the different arrays and identified potential outliers. ANOVA with a nominal alpha value set to 0.05 was then used to determine those probe sets significantly different between the sample set defined variables including average daily gain (ADG), meat score index (MSA), disease status (resilient or susceptible), time point of sampling, and nutrition or stress source. Gene sets were combined, followed by a Multiple testing correction to reduce the false positive rate (Hochberg & Benjamini, 1990). To ensure the accuracy of the results, only consistently expressed differentially expressed genes with a false-discovery rate (FDR) ≤0.05, a fold change (FC) ≤-1.5 or ≥ 1.5 were included as potential genes of interest.

Functional and pathway analysis of genes of interest

Gene lists were prepared from each comparison group and identification of cellular functions of differentially regulated genes was achieved utilising enrichment analysis within the Partek pathway analysis tool. Enrichment analysis is a technique commonly used to add biological context to a list of genes, such as a list of significant genes. The procedure is based on assigning genes to groups and then finding overrepresented groups in filtered gene lists, using a Fisher's exact test. The higher the

enrichment score, the more overrepresented the pathway is within the input list of significant genes. An enrichment score of three is equal to a p-value of 0.05. Within Partek pathway analysis, the significantly enriched functional categories were derived from Gene Ontology (Ashburner et al., 2000), KEGG databases (Kanehisa, 1997) and the Ingenuity® Knowledge Base (http://www.ingenuity.com/science/knowledge-base). A stringent cut-off value was applied to this analysis and the functional categories highlighted are all significant, with a high fold enrichment score paired with a low p-value.

Following this basic bioinformatic analysis deeper pathway and association mining was carried out using QIAGEN's Ingenuity® Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). Within the IPA framework the Partek software generated ANOVA files were analysed to identify statistically relevant genes of interest. These modified gene lists were subjected to core analysis for identification of upstream regulators, functional analysis to identify functional groups associated with genes, defined by outcome and finally, and biomarker analysis to identify genes that may be of potential use as biomarkers of immune function. The results of all these tests were cross compared to ensure consistency.

3.4.5.3   qPCR validation of correlates

Data collected from the qRT-PCR were analysed utilising a modified Comparative Ct ($\Delta\Delta$Ct) method (Livak & Schmittgen, 2001).

3.4.5.4   Telomere length

The RTL measurements at different timepoints for the same individual animals were compared using a paired t-test. Sample RTL values when graphed showed a skewed distribution to the left and hence a log10 transformation was applied to achieve a normal distribution. The effects of RTL compared to the disease or production outcomes were assessed using a non-parametric Spearman's correlation analysis and a one-way analysis of variance (ANOVA) to determine if there was a significant difference between variables such as Breed, Sex or initial weight. All statistical analyses were performed in GraphPad Prism (version 8.1.2).

4. Results

4.1 Animal models

4.1.1 MAP and *H.contortus* co-challenge trial

All of the animals in the co-challenge trial experienced at least a mild disease event from either JD, Barbers Pole Worm, or both. Only a single animal did not show signs of paratuberculosis lesions upon necropsy; this animal also achieved the highest average daily weight gain of the group. Within this trial, 88% of animals developed multibacillary paratuberculosis lesions, with 52% of animals experiencing severe paratuberculosis disease. Similarly, 70% of the animals displayed high to very high faecal egg burdens (up to 10,000 egg/gram), and 55% of the animals had moderate to poor average daily weight gain results. There were animals that could be classified as resilient to paratuberculosis and/or *H.contortus* infection, based on their disease burden metrics (see Table 4.1.1).

Table 4.1.1: Average faecal egg counts, paratuberculosis score, average daily gain and category groupings for the co-challenge trial animals.

Animal Number	Measures			Category		
	AFEC	ParaTb score	ADG	AFEC	ParaTb score	ADG
1	1655	89.5	6.9	Intermediate	Susceptible	poor
2	4615	80	11.5	Susceptible	Susceptible	mid
3	8825	92	21.5	Susceptible	Susceptible	mid
4	2118	34	27.8	Intermediate	Intermediate	mid
5	1683	10	50.1	Intermediate	Resilient	good
6*	919	83	na	Resilient	Susceptible	na
7	1174	36	32.2	Resilient	Intermediate	good
8	280	60	15.6	Resilient	Intermediate	mid
9	3570	89.5	9.9	Intermediate	Susceptible	poor
10	1224	89	26.8	Intermediate	Susceptible	mid
11	705	41.5	41.2	Resilient	Intermediate	good
12	3238	66.5	23.1	Intermediate	Intermediate	mid
13	3450	66	28.7	Intermediate	Intermediate	mid
14	175	83	30.6	Resilient	Susceptible	good
15	5125	45	23.1	Susceptible	Intermediate	mid
16	5475	87.5	14.2	Susceptible	Susceptible	mid

17	3325	78	3.1	Intermediate	Intermediate	poor
18	1361	90.5	26.4	Intermediate	Susceptible	mid
19	6675	92	19.0	Susceptible	Susceptible	mid
20	1017	92.5	16.3	Resilient	Susceptible	mid

ADG: Average daily gain; AFEC: Average faecal worm egg count. ParaTb: paratuberculosis score. (see **Appendix 8.5** for additional details)

4.1.2 **Salmonella challenge trial**

Within the Salmonella challenge trial, four animals developed severe Salmonellosis and were euthanased at Day 7 post-challenge. These were categorised as Susceptible and had very high colony counts from faeces (average > 500,000). The remaining animals could be classified as either Resilient or Intermediate, depending on the faecal Salmonella culture results (see Table 4.1.2).

Table 4.1.2: Salmonella colony counts and category grouping.

| KLN | RTL | Faecal culture colony counts | | | | Category* |
		Day 3	Day 7	Day 10	Average	
11	1.233	5.9	6.1	0	4	Resilient
36	1.181	6.6	6.1	0	4	Resilient
1	0.807	459	6.3	0	155	Resilient
9	1.151	55	360	423	280	Resilient
30	1.026	397	1170	54.6	541	Intermediate
16	1.424	1440	431	5.2	626	Intermediate
5	0.640	3791	6.4	0	1266	Intermediate
7	0.604	3625	29294	56	10992	Intermediate
18	0.982	30606	195715	0	75440	Intermediate
14	1.060	1191308	19800	6.1	403705	Intermediate
12	1.151	686	1416470	258	472472	Intermediate
15	1.795	20310	3010000	-	1515155	Susceptible
3	0.677	315000	10537864	-	5426432	Susceptible
6	0.666	31500	14718750	-	7375125	Susceptible
2	0.756	44521	22130357	-	11087439	Susceptible

*Category classifications. Resilient: average colony count < 500; Intermediate: average colony count 500 to <500,000; Susceptible: culled at Day 7, average colony count > 500,000.

4.1.3 Commercial property sourced sheep (Arthursleigh)

Repeat sampling was not possible for all 220 sheep. Of the 116 animals (Table 4.1.3) where it was possible to collect a repeat sample, 12 were categorised as low weight gain, 116 as mid weight gain and 57 achieved a high weight over the duration of the sampling period (210 days).

Due to the 2019-2020 Bushfires, the February 2020 flash floods and the Coronavirus pandemic, it was not possible to collect samples at all the planned time points.

Table 4.1.3: Production outcomes (by weight) of sheep managed under commercial livestock rearing protocols.

Key List #	total weight gain (kg)	ADG (kg)	% weight gain	Category*
28	8	0.04	25	low
203	10	0.05	28	low
169	11	0.05	32	low
56	11	0.05	33	low
72	10	0.05	33	low
11	12	0.06	38	low
66	14	0.07	40	low
75	13	0.06	41	low
17	12	0.06	41	low
38	15	0.07	42	low
131	15	0.07	44	low
140	14	0.07	45	low
55	15	0.07	47	mid
117	15	0.07	47	mid
48	17	0.08	47	mid
83	18	0.09	47	mid
51	16	0.08	48	mid
77	18	0.09	49	mid
122	19	0.09	51	mid
185	17	0.08	52	mid
190	19	0.09	53	mid
135	18	0.09	53	mid
22	16	0.08	53	mid
23	16	0.08	53	mid
69	16	0.08	53	mid
84	16	0.08	53	mid
214	15	0.07	54	mid
158	19	0.09	54	mid
4	17	0.08	55	mid
7	17	0.08	55	mid
39	17	0.08	55	mid

Key List #	total weight gain (kg)	ADG (kg)	% weight gain	Category*
62	16	0.08	55	mid
159	20	0.10	56	mid
59	19	0.09	56	mid
85	18	0.09	56	mid
137	17	0.08	57	mid
94	19	0.09	58	mid
26	18	0.09	58	mid
164	18	0.09	58	mid
205	20	0.10	59	mid
74	19	0.09	59	mid
99	18	0.09	60	mid
114	18	0.09	60	mid
130	18	0.09	60	mid
144	18	0.09	60	mid
173	18	0.09	60	mid
194	20	0.10	61	mid
183	17	0.08	61	mid
98	19	0.09	61	mid
40	16	0.08	62	mid
93	21	0.10	62	mid
71	20	0.10	63	mid
168	20	0.10	63	mid
153	17	0.08	63	mid
82	21	0.10	64	mid
91	21	0.10	64	mid
95	21	0.10	64	mid
120	21	0.10	64	mid
21	18	0.09	64	mid
218	18	0.09	64	mid
86	20	0.10	65	mid
181	20	0.10	65	mid
187	20	0.10	65	mid
101	22	0.10	65	mid
13	15	0.07	65	mid
105	17	0.08	65	mid
41	19	0.09	66	mid
60	19	0.09	66	mid
134	19	0.09	66	mid
12	18	0.09	67	mid
19	20	0.10	67	mid
42	20	0.10	67	mid
103	18	0.09	67	mid
138	20	0.10	67	mid
47	23	0.08	68	mid

Key List #	total weight gain (kg)	ADG (kg)	% weight gain	Category*
108	21	0.10	68	mid
192	21	0.10	68	mid
9	19	0.09	68	mid
79	19	0.12	68	mid
141	19	0.09	68	mid
211	19	0.09	68	mid
1	17	0.08	68	mid
80	22	0.10	69	mid
154	20	0.10	69	mid
170	20	0.10	69	mid
46	25	0.12	69	mid
63	24	0.11	71	mid
30	22	0.10	71	mid
8	20	0.10	71	mid
87	20	0.10	71	mid
125	23	0.11	72	mid
201	23	0.11	72	mid
127	18	0.09	72	mid
133	21	0.10	72	mid
212	21	0.10	72	mid
172	22	0.10	73	mid
178	22	0.10	73	mid
220	22	0.10	73	mid
57	17	0.08	74	mid
35	20	0.10	74	mid
61	20	0.10	74	mid
166	20	0.10	74	mid
193	20	0.10	74	mid
191	23	0.11	74	mid
24	21	0.10	75	mid
113	24	0.11	75	mid
126	18	0.09	75	mid
128	24	0.11	75	mid
182	24	0.11	75	mid
202	21	0.10	75	mid
31	19	0.09	76	mid
132	19	0.09	76	mid
53	23	0.11	77	mid
3	20	0.10	77	mid
44	20	0.10	77	mid
110	20	0.10	77	mid
142	20	0.10	77	mid
179	20	0.10	77	mid
104	27	0.13	77	mid

Key List #	total weight gain (kg)	ADG (kg)	% weight gain	Category*
119	21	0.10	78	mid
149	21	0.10	78	mid
186	21	0.10	78	mid
116	22	0.10	79	mid
174	22	0.10	79	mid
213	19	0.09	79	mid
54	23	0.11	79	mid
199	23	0.11	79	mid
15	27	0.13	79	mid
88	24	0.11	80	high
209	20	0.10	80	high
81	25	0.12	81	high
89	25	0.12	81	high
10	21	0.10	81	high
97	21	0.10	81	high
195	26	0.12	81	high
208	22	0.10	81	high
197	23	0.11	82	high
207	24	0.11	83	high
196	25	0.12	83	high
76	21	0.10	84	high
124	21	0.10	84	high
32	22	0.10	85	high
123	22	0.10	85	high
2	23	0.11	85	high
50	23	0.11	85	high
78	23	0.11	85	high
150	26	0.12	87	high
68	22	0.10	88	high
161	23	0.11	88	high
204	23	0.11	88	high
96	24	0.11	89	high
107	25	0.12	89	high
143	25	0.12	89	high
215	28	0.13	90	high
109	22	0.10	92	high
18	23	0.11	92	high
102	23	0.11	92	high
14	24	0.11	92	high
43	24	0.11	92	high
58	24	0.11	92	high
90	24	0.11	92	high
167	26	0.12	93	high
171	26	0.12	93	high

Key List #	total weight gain (kg)	ADG (kg)	% weight gain	Category*
155	24	0.11	96	high
175	24	0.11	96	high
180	26	0.12	96	high
5	27	0.13	96	high
67	30	0.14	97	high
20	31	0.15	100	high
27	29	0.14	100	high
100	26	0.12	100	high
112	27	0.13	100	high
139	25	0.12	100	high
184	29	0.14	104	high
29	27	0.13	104	high
118	26	0.12	104	high
217	26	0.12	104	high
162	29	0.14	107	high
70	27	0.13	113	high
177	32	0.15	114	high
129	30	0.14	115	high
25	33	0.16	118	high
219	30	0.14	120	high
157	29	0.14	121	high
6	30	0.14	143	high

*Category classifications. High weight gain ≥80 % gain, Mid weight gain between 47 and 79% gain and Low weight gain ≤ 45% weight gain.

4.1.4 *Mycobacterium avium*. subspecies *paratuberculosis* sheep exposure trial (project P.PSH.0311)

Twenty-five of the trial animals were repeat sampled for transcriptomic analysis (Table 4.1.4). resulting in 125 arrays. Disease susceptibility/resilience outcomes are reported in Table 4.1.5.

Table 4.1.4: Animal sampling across the time points in trial P.PSH.0311.1. Data are the number of animals and gene chips used.

Group	Months post exposure to MAP				
	2	4	6	9	12
Control	5	5	5	5	5
MAP exposed	20	20	20	20	20

Table 4.1.5: Sample information file defining the disease associated classification outcomes for each animal within the Merino sheep trial PPSH 0311.1 used for gene expression analysis with Affymetrix GeneChip Bovine Genome Array.

Key List	Treatment	Month of death post MAP	Survival (months)	Event	Faecal Culture	Histopathology grade[a]	Disease severity (Perez)	Non-infected /infected[b]	Resistant /susceptible	Non-infectious /infectious
1	Unexposed	20	50	control	0	0	Control	Control	Control	Control
2	Unexposed	20	50	control	0	0	Control	Control	Control	Control
3	Unexposed	20	50	control	0	0	Control	Control	Control	Control
4	Unexposed	20	50	control	0	0	Control	Control	Control	Control
5	Unexposed	14	50	control	0	0	Control	Control	Control	Control
11	MAP	20	50	lived	0	0	Recovered	non-infected	resistant	non-infectious
12	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
13	MAP	14	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
14	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
15	MAP	14	14	died	1	3a-c	Paucibacillary	Infected	susceptible	infectious
16	MAP	14	14	died	1	3a-3c	Paucibacillary	Infected	susceptible	infectious
17	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
18	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
19	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
20	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
21	MAP	14	14	died	1	3a-c	Paucibacillary	Infected	susceptible	infectious
22	MAP	14	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
23	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
24	MAP	12	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
25	MAP	14	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
26	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
27	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
28	MAP	17	17	died	1	3b	Multibacillary	Infected	susceptible	infectious
29	MAP	20	20	died	1	3b	Multibacillary	Infected	susceptible	infectious
30	MAP	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious

[a] Severity of disease defined by histopathological lesion score using established criteria (Perez, Garcia Marin & Badiola, 1996) [b] Inoculated animals were classified as infected if any tissue at necropsy was culture positive for MAP

4.1.5 *Mycobacterium avium* subspecies *paratuberculosis* exposure trial in vaccinated sheep (project P.PSH.0576)

Thirty-four of the trial animals were sampled for transcriptomic analysis. Johne's disease susceptibility/resilience outcomes are reported in Table 4.1.6.

Table 4.1.6. Disease outcomes for Gudair® vaccinated Merino sheep

Key List number*	Exposure status	Severity†	Infection status‡
124	Control	Control	Control
125	Control	Control	Control
126	Control	Control	Control
133	Control	Control	Control
135	Infected	Recovered	non-infected
137	Infected	Recovered	non-infected
139	Infected	Paucibacillary	Infected
140	Infected	Multibacillary	Infected
141	Infected	Recovered	non-infected
142	Infected	Recovered	non-infected
144	Infected	Recovered	non-infected
145	Infected	Recovered	non-infected
148	Infected	Recovered	non-infected
149	Infected	Paucibacillary	Infected
150	Infected	Paucibacillary	Infected
153	Infected	Recovered	non-infected
2001	Control	Control	Control
2002	Control	Control	Control
2007	Infected	Paucibacillary	Infected
2009	Infected	Paucibacillary	Infected
2010	Infected	Recovered	non-infected
2011	Infected	Recovered	non-infected
2012	Infected	Recovered	non-infected
2013	Infected	Recovered	non-infected
2015	Infected	Recovered	non-infected
2016	Infected	Recovered	non-infected
2017	Infected	Recovered	non-infected
2018	Infected	Recovered	non-infected
2019	Infected	Recovered	Infected
2020	Infected	Paucibacillary	non-infected
2022	Infected	Recovered	non-infected
2023	Infected	Paucibacillary	non-infected
2024	Infected	Recovered	non-infected
2025	Infected	Recovered	non-infected

* Identification number of individual animals.
† Severity of disease defined by histopathological lesion score using established criteria (Perez, Garcia Marin & Badiola, 1996).
‡ Inoculated animals were classified as infected if any tissue at necropsy was culture positive for MAP.

### 4.1.6 Brisbane Valley ACC feedlot 78 day short-finished cattle

Forty-nine cattle were sampled in a feedlot, sourced from four different farms. There were 23 females (average weight at entry = 366 kg (333 to 417 kg)) and 26 males (average weight at entry = 375 kg (343 to 414 kg)) of varying breeds, as shown in Tables 4.1.7 and 4.1.8.

Table 4.1.7. Breed, gender and weight demographics of cattle included in the Feedlot trial

Breed	Female		Male	
	Number	Av. Weight (kg)	Number	Av. Weight (kg)
Angus	0	-	1	394
Braford	1	349	0	-
Brahman	0	-	1	381
Brangus	3	372	0	-
Charbray	8	360	6	376
Charolais	5	386	6	371
Droughtmaster	3	349	7	377
Hereford	0	-	1	396
Murray Gray	0	-	3	360
Red Angus	1	-	0	394
Santa Gertrudis	0	-	1	379

For two female cattle, the breed was not recorded. The weights of these animals were 333 kg and 352 kg.

Over the feedlot finishing period, all animals gained weight, however there was a broad range of performance outcomes in terms of average daily gain and weight gain proportional to the entry weight (% weight gain) (Fig. 4.1.1).
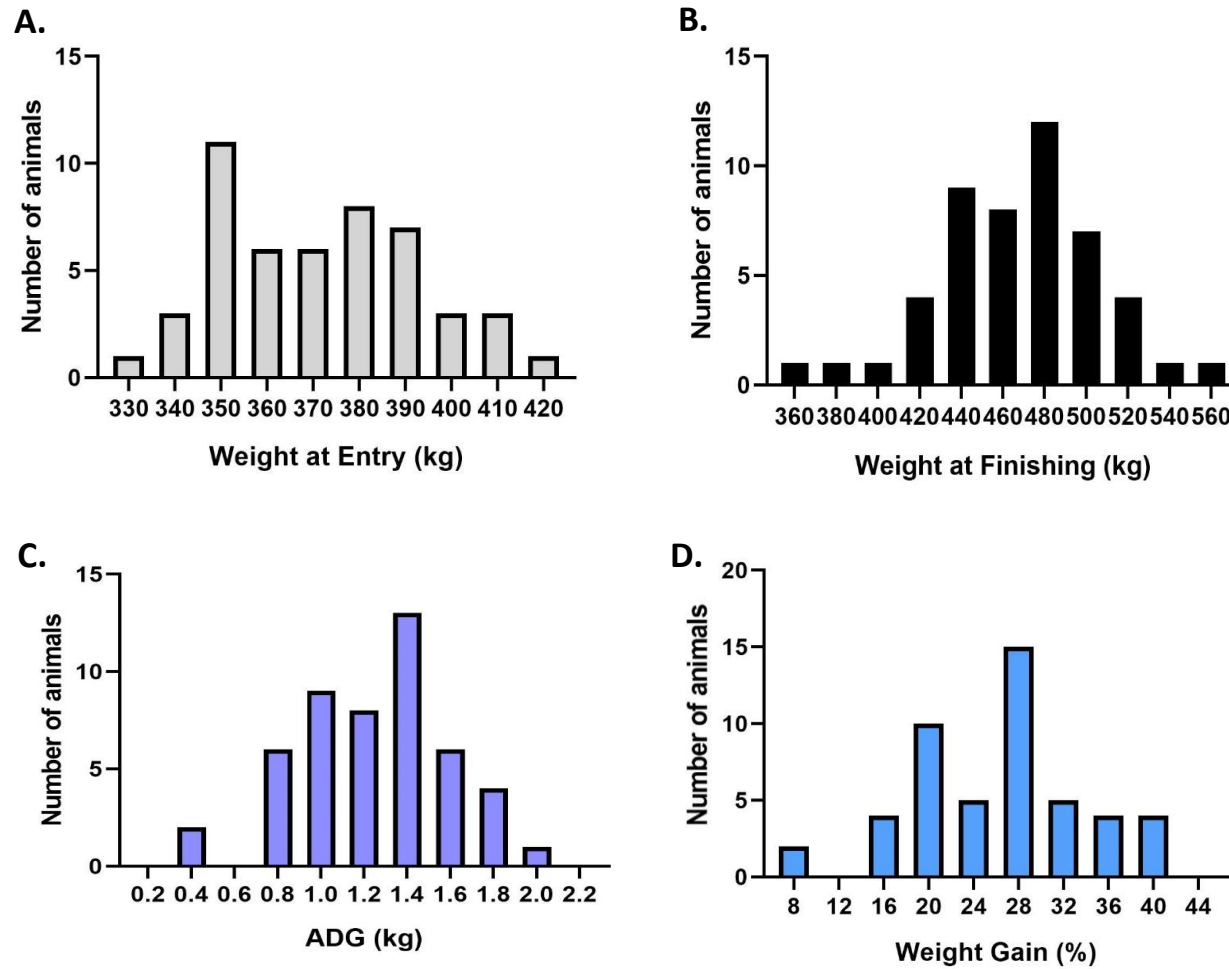
Figure 4.1.1. Frequency histograms of A. Weights at entry, B. Weights at Finishing, C. Average Daily weight gain (ADG) and D. weight gain proportional to the entry weight (Weight Gain (%))

Thirteen cattle were identified as high weight gain achievers, 20 cattle were within the low percentage weight gain and the remaining cattle were classified as mid-range (n=16) (Table 4.1.8). Analysis revealed a correlation (p=0.0467) between sex and ADG/PWG but no correlation between MSA or breed (*Bos taurus* or *Bos indicus* cross). Hereafter the variable ADG is used to describe weight gain.
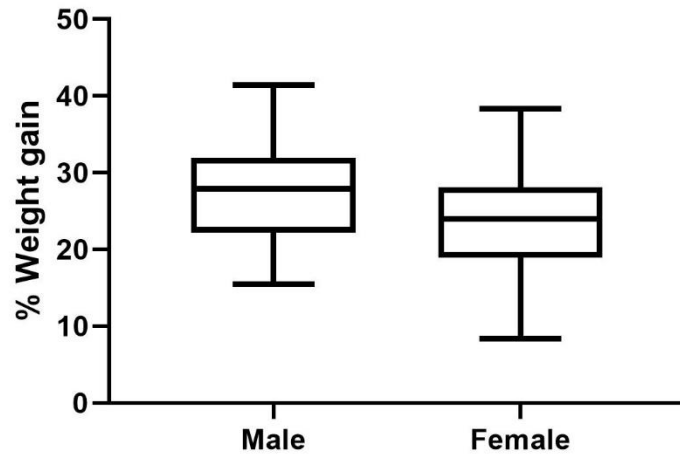
Figure 4.1.2. Correlation between percentage weight gain and gender.

Table 4.1.8. Demographics for 49 individual cattle sampled during the trial. Animals selected for transcriptomic analysis are indicated.

| Cow ID	Farm ID	Sex	Bos breed identity	Selected for GeneChip™ Array	weight at induction (kg)	Weight pre-slaughter (kg)	ADG (kg)	% weight change from induction	Weight gain range	MSA Index score	MSA range
13892	1	F	Bos Indicus x Bos Taurus		387	478	1.17	24	Low	53	Bottom 25%
13895	1	F	Bos Indicus x Bos Taurus		351	457	1.36	30	High	53	Bottom 25%
13883	1	F	Bos Indicus x Bos Taurus		345	434	1.14	26	Mid	54	Bottom 25%
13880	1	F	Bos Taurus	Yes	417	478	0.78	15	Low	55	Bottom 25%
13877	1	F	Bos Indicus x Bos Taurus		358	431	0.94	20	Low	56	Bottom 25%
13879	1	F	Bos Taurus		386	497	1.42	29	Mid	57	Average
13887	1	F	Bos Indicus x Bos Taurus	Yes	365	438	0.94	20	Low	57	Average
13890	1	F	Bos Indicus x Bos Taurus		365	467	1.31	28	Mid	58	Average
13888	1	F	Bos Taurus		351	446	1.22	27	Mid	59	Average
13882	1	F	Bos Indicus x Bos Taurus	Yes	345	407	0.79	18	Low	60	Top 25%
13891	1	F	Bos Indicus x Bos Taurus		343	432	1.14	26	Mid	60	Top 25%
13884	1	F	Bos Taurus		399	503	1.33	26	Mid	60	Top 25%
13896	1	F	Bos Indicus x Bos Taurus		350	423	0.94	21	Low	60	Top 25%
13885	1	F	Bos Indicus x Bos Taurus	Yes	379	515	1.74	36	High	60	Top 25%
13894	1	F	Bos Indicus x Bos Taurus		352	383	0.43	9	Very low		Not recorded
13907	2	F	Bos Indicus x Bos Taurus		352	424	0.92	20	Low	52	Bottom 25%
13898	2	F	Bos Taurus		379	470	1.17	24	Low	54	Bottom 25%
13903	2	M	Bos Indicus x Bos Taurus		358	439	1.04	23	Low	55	Bottom 25%
13904	2	M	Bos Indicus x Bos Taurus		356	430	0.95	21	Low	57	Average
13908	2	F	Bos Indicus x Bos Taurus	Yes	342	473	1.68	38	High	58	Average
13899	2	F	Bos Indicus x Bos Taurus		349	415	0.85	19	Low	59	Average

Cow ID	Farm ID	Sex	Bos breed identity	Selected for GeneChip™ Array	weight at induction (kg)	Weight pre-slaughter (kg)	ADG (kg)	% weight change from induction	Weight gain range	MSA Index score	MSA range
13902	2	M	Bos Indicus x Bos Taurus	Yes	385	461	0.97	20	Low	60	Top 25%
13910	2	M	Bos Indicus x Bos Taurus	Yes	381	440	0.76	15	Low	61	Top 25%
13901	2	M	Bos Taurus		396	478	1.05	21	Low	61	Top 25%
13906	2	F	Bos Indicus x Bos Taurus		333	361	0.39	8	low		Not recorded
13837	3	M	Bos Indicus x Bos Taurus		399	525	1.62	32	High	53	Bottom 25%
13834	3	M	Bos Taurus	Yes	350	487	1.76	39	High	54	Bottom 25%
13868	3	M	Bos Taurus		391	501	1.41	28	Mid	54	Bottom 25%
13833	3	M	Bos Indicus x Bos Taurus	Yes	379	445	0.85	17	Low	56	Bottom 25%
13862	3	M	Bos Indicus x Bos Taurus	Yes	386	546	2.05	41	High	57	Average
13855	3	M	Bos Indicus x Bos Taurus		377	488	1.42	29	Mid	57	Average
13843	3	M	Bos Taurus		357	461	1.33	29	Mid	58	Average
13873	3	M	Bos Indicus x Bos Taurus	Yes	351	486	1.73	38	High	58	Average
13860	3	M	Bos Taurus		373	479	1.36	28	Mid	59	Average
13864	3	M	Bos Taurus	Yes	374	503	1.65	34	High	59	Average
13841	3	M	Bos Indicus x Bos Taurus		375	494	1.53	32	High	59	Average
13850	3	M	Bos Taurus		369	489	1.54	33	High	59	Average
13839	3	M	Bos Indicus x Bos Taurus		381	474	1.19	24	Low	60	Top 25%
13866	3	M	Bos Indicus x Bos Taurus		414	524	1.41	27	Mid	60	Top 25%
13846	3	M	Bos Taurus		394	503	1.40	28	Mid	61	Top 25%
13844	3	M	Bos Taurus		343	451	1.38	31	High	61	Top 25%
13854	3	M	Bos Indicus x Bos Taurus		373	467	1.21	25	Mid	61	Top 25%
13861	3	M	Bos Taurus		394	498	1.33	26	Mid	61	Top 25%

Cow ID	Farm ID	Sex	Bos breed identity	Selected for GeneChip™ Array	weight at induction (kg)	Weight pre-slaughter (kg)	ADG (kg)	% weight change from induction	Weight gain range	MSA Index score	MSA range
13852	3	M	Bos Indicus x Bos Taurus		380	458	1.00	21	Low	61	Top 25%
13849	3	M	Bos Taurus		355	475	1.54	34	High	62	Top 25%
13872	3	M	Bos Indicus x Bos Taurus		358	451	1.19	26	Mid		Not recorded
13915	4	F	Bos Indicus x Bos Taurus		413	529	1.49	28	Mid	57	Average
13916	4	F	Bos Indicus x Bos Taurus		352	411	0.76	17	Low	59	Average
13912	4	F	Bos Taurus		414	558	1.85	35	High	59	Average

On arrival to the feedlot there was no significant difference between the weights of the cattle sourced from individual farms however, during the subsequent weighing events, cattle from Farm 1 and 3 responded well to the transition from grazing and exposure to the social and physiological stresses inherent with the feedlot environment (Table 4.1.9, Fig. 4.1.3). Weight gain of cattle from Farm 2 was significantly different from Farms 1 and 3 ($p \leq 0.005$), whilst cattle obtained from Farm 3 gained the most weight over the finishing period (Table 4.1.9) and exhibited rapid acclimatisation to the feedlot environment (Figure 4.1.4). These data suggest a farm of origin effect.

Table 4.1.9. Production metrics by farm of origin.

Farm #	Animals (n=)		Weight (kg)				ADG since induction	Average weight gain (kg)	Average % gain
	Male	Female	Induction	Post acclimatisation	Pre lairage	Pre-slaughter			
1	0	15	367	410	447	458	1.16	90.4	24.6
2	5	5	366	391	433	448	1.04	81.3	22.3
3	22	0	380	434	481	493	1.45	112.93	29.78
4		3	383	414	458	470	1.12	87.5	22.42
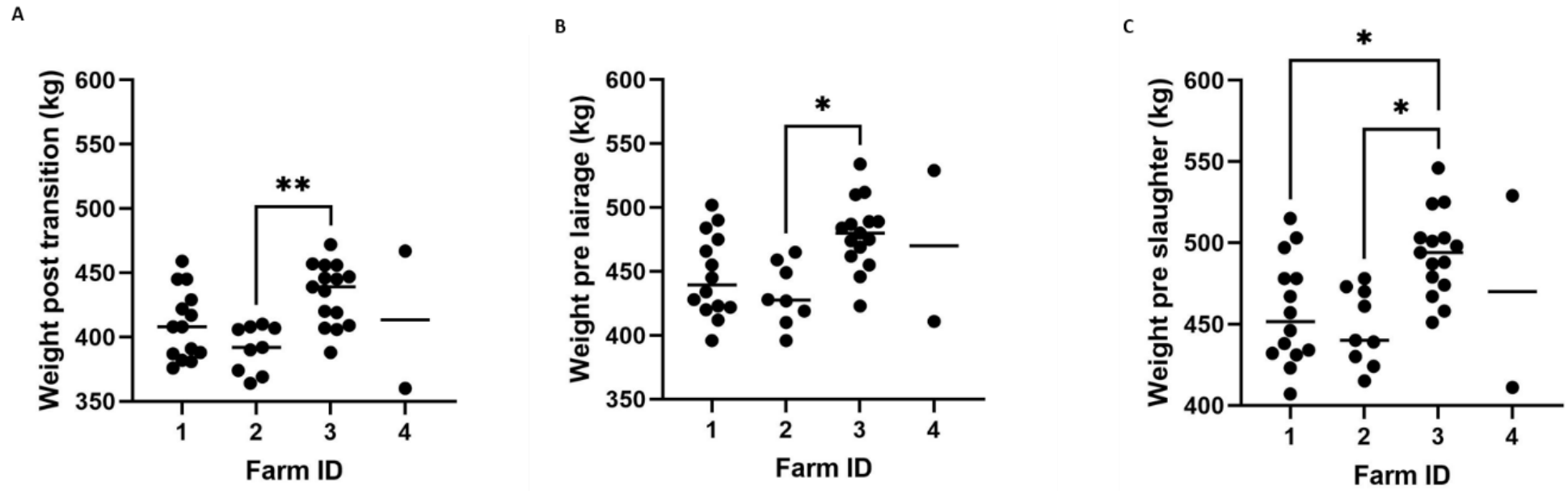
Figure 4.1.3. Weight distribution of individual cows by farm. * p≤ 0.005 and ** p≤0.0005

Cattle from Farm 2 also exhibited low ADG during the transition period possibly indicative of poor acclimatisation to the feedlot setting (Fig. 4.1.4).
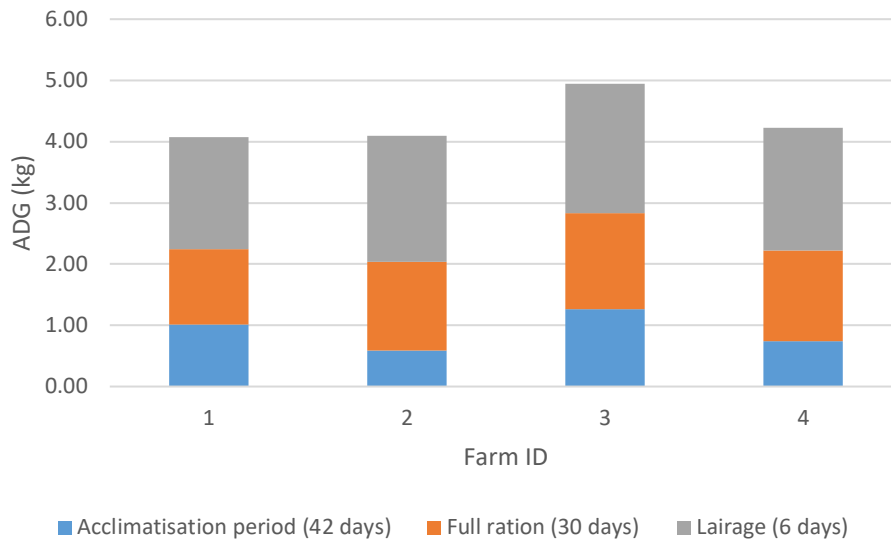
Figure 4.1.4. Average daily gain between sample collection points by farm.

4.1.7 *Mycobacterium avium* sub-species *paratuberculosis* experimental trial (project P.PSH.0297)

Twenty-five of the trial animals were repeat sampled for transcriptomic analysis (Table 4.1.10). resulting in 125 arrays. Disease susceptibility/resilience outcomes are reported in Table 4.1.11.

Table 4.1.10: Animal sampling across the time points in trial P.PSH.0297.2. Data are the number of animals and gene chips used.

Group	Months post exposure to MAP				
	2	**4**	**6**	**9**	**12**
Control	5	5	5	5	5
MAP exposed	20	20	20	20	20

Table 4.1.11: Classification of cattle from trial P.PSH.0297.2

Key List #	MAP status	Histopathology outcome (Perez score)	IFNγ at 4 months post exposure
1	unexposed	control	control
2	unexposed	control	control
3	unexposed	control	control
4	unexposed	control	control
5	unexposed	control	control
11	exposed	NVLS	high
12	exposed	NVLS	high
13	exposed	NVLS	high
14	exposed	NVLS	low
15	exposed	NVLS	low
16	exposed	NVLS	high
17	exposed	early Paucibacillary	low
18	exposed	NVLS	low
19	exposed	NVLS	low
20	exposed	NVLS	high
21	exposed	NVLS	high
22	exposed	NVLS	low
23	exposed	early Paucibacillary	low
24	exposed	NVLS	low
25	exposed	NVLS	high
26	exposed	NVLS	low
27	exposed	NVLS	high
28	exposed	NVLS	high
29	exposed	NVLS	high
30	exposed	NVLS	low

NVLS – no visible lesions

4.1.8 Novel vaccine trial (Camden, NSW)

Weights of Angus steers in the cattle vaccine trial were taken at regular intervals during the 5-month duration of the trial (Fig. 4.1.5). There was no significant variation in weight between groups.
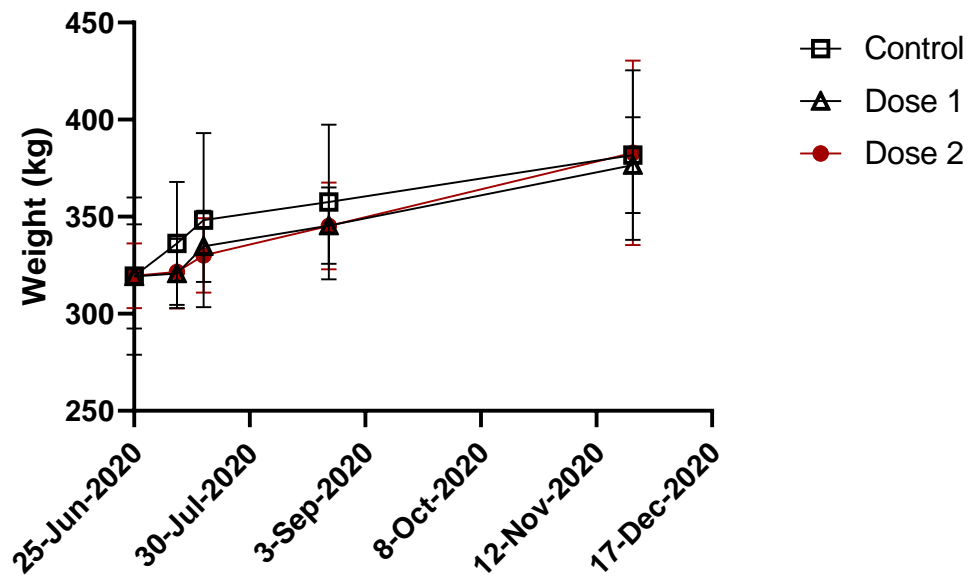

Figure 4.1.5. Weights of cattle in the novel vaccine trial

4.1.9 Bushfire exposed cattle

The cattle included in the study were from two farms; a fire-affected farm (BF1-24) and a control farm (BF1-1), shown in Table 4.1.12.

Table 4.1.12: Burn status, sex and breed of cattle from the two farms investigated.

Trial	Farm ID	KL	Fire exposure	Sex	Breed
Bushfire	BF1-1	1	none	M	Angus
Bushfire	BF1-1	2	none	M	Angus
Bushfire	BF1-1	3	none	M	Angus
Bushfire	BF1-1	4	none	M	Angus
Bushfire	BF1-1	5	none	M	Angus
Bushfire	BF1-1	6	none	M	Angus
Bushfire	BF1-24	1	Severe burns	F	Angus cross
Bushfire	BF1-24	2	Severe burns	M	Angus
Bushfire	BF1-24	3	Severe burns	F	Angus

Trial	Farm ID	KL	Fire exposure	Sex	Breed
Bushfire	BF1-24	4	Unharmed	F	Angus
Bushfire	BF1-24	5	Unharmed	F	Angus
Bushfire	BF1-24	6	Unharmed	F	Angus
Bushfire	BF1-24	7	Unharmed	M	Dairy cross
Bushfire	BF1-24	8	Unharmed	M	Dairy cross
Bushfire	BF1-24	9	Severe burns	F	Angus
Bushfire	BF1-24	10	Unharmed	F	Angus
Bushfire	BF1-24	11	Unharmed	F	Angus
Bushfire	BF1-24	12	Unharmed	F	Angus

4.2 Identification of immune factors

4.2.1 Stimulation assay

The mammalian immune system is a complex, interconnected combination of pathways orchestrated by innate cells (e.g. neutrophils, macrophages) which are the frontline protectors, and adaptive immune cells (lymphocytes) which assist in eliminating pathogens. Innate immune cells recognise conserved patterns on microbes (Pathogen Associated Molecular Patterns, PAMPs) through pathogen recognition receptors such as the family of Toll-like Receptors (TLR). Lymphocyte activation is based on a more restricted recognition pattern with each cell only able to bind to one specific molecular structure (antigen). In addition, activated lymphocytes undergo clonal proliferation which increases the number of these antigen-specific cells to allow a more potent and specific secondary immune response when the same pathogen is encountered again. Immune responses between individuals are highly variable and accounts for the spectrum of disease outcomes observed when subjected to similar pathogen burden (de Silva et al., 2018). This variability can be due to multiple factors that may be biological, genetic, or environmental in nature. We hypothesised that the inherent capacity of an individual's innate and adaptive immune cells to respond to challenge (e.g. infection, environmental stress) could reflect their ability to maintain overall health. To test this, we collected blood samples from animals under normal husbandry practices on-farm or under experimental pathogen challenge and assessed cytokine responses to stimulation with immune pathway-specific stimuli as well as pathogen-specific stimuli. Others have attempted to characterise a healthy human immune response in relation to developing better vaccines and treatments using whole blood stimulation assays (Duffy et al., 2014). The stimulants used (Table 4.2.1) target specific cellular receptors and activate downstream pathways. Cytokines are cell-cell signalling proteins that play important effector roles in immune pathways. Cytokine

results were then analysed based on production outcomes such as weight gain or susceptibility to disease.

Regulation of cellular processes by cytokines is often a complex, dynamic process involving multiple proteins. Therefore, the ability to simultaneously detect multiple cytokines, achieved by protein arrays was a useful tool in the initial screening process in a subset of animals to determine which cytokines could be potentially relevant to discriminating between production outcome groups. Select cytokines were then assessed in a larger cohort of animals.

Table 4.2.1. Stimuli selected to assess response capacity of immune cells

| Stimulus	Target pathway	Source
Pokeweed mitogen (PWM)	Lymphocyte proliferation via binding to membrane glycoproteins	Lectin from *Phytolacca americana*
Concanavalin A (Con A)	T-lymphocyte proliferation via binding to mannose and glucose moieties	Protein from *Canavalia ensiformis*
Lipopolysaccharide (LPS)	TLR2 and TLR4; activates secretion of pro-inflammatory cytokines	From *Escherichia coli*, has other bacterial components including lipoproteins
Pam3CysSerLys4 (Pam3CSK4)	TLR2/TLR1; potent pro-inflammatory activator	Synthetic triacylated lipopeptide
Polyinosinic-polycytidylic acid (poly(I:C))	TLR3; simulates viral infection	Synthetic analogue of double-stranded RNA
Flagellin (FLA-ST)	TLR5; triggers pro-inflammatory responses	Flagellin from *Salmonella typhimurium*
H. contortus antigen (H)	Memory to previous *H. contortus* encounter	*H. contortus* L3 larvae
316v (MAP antigen)	Memory to previous MAP encounter	*M. avium* subspecies *paratuberculosis*

4.2.2 Initial screening by protein array

4.2.2.1 Baseline measures

Cytokine responses were screened in blood samples collected on-farm, prior to animals being transported for an experimental trial with exposure to a single pathogen (section 3.1.2). This was considered a 'baseline' sample with animals subjected to minimal stress. Sheep were grouped based on disease outcome at the end of the trial. For this initial assessment 4 animals that succumbed to infection (susceptible) and 3 that survived exposure to Salmonella (resilient) were selected. Several

cytokines had to be removed from further analysis due to not meeting assay criteria. Results for 12 of 20 cytokines in the array are shown in **Appendix 8.1.**

For IL-8 and IL-21 (Fig. 4.2.1) disease outcome was a significant effect ($p<0.05$) but there were no significant differences between the outcome groups for any particular stimulant. For LPS-induced CXCL10 there was a significant difference between the susceptible and resilient animals (Fig. 4.2.1) in this sample taken prior to animals being subjected to transport and re-homing stresses, change in feed (grass-fed to chaff) and exposure to pathogen. LPS and PWM stimulated IL-17 tended to be lower in resilient sheep. ConA and PWM stimulated IL-4 tended to be higher in the resilient group. From this initial screening, IL-8, IL-21 and CXCL10 were selected as suitable cytokines for further assessment.
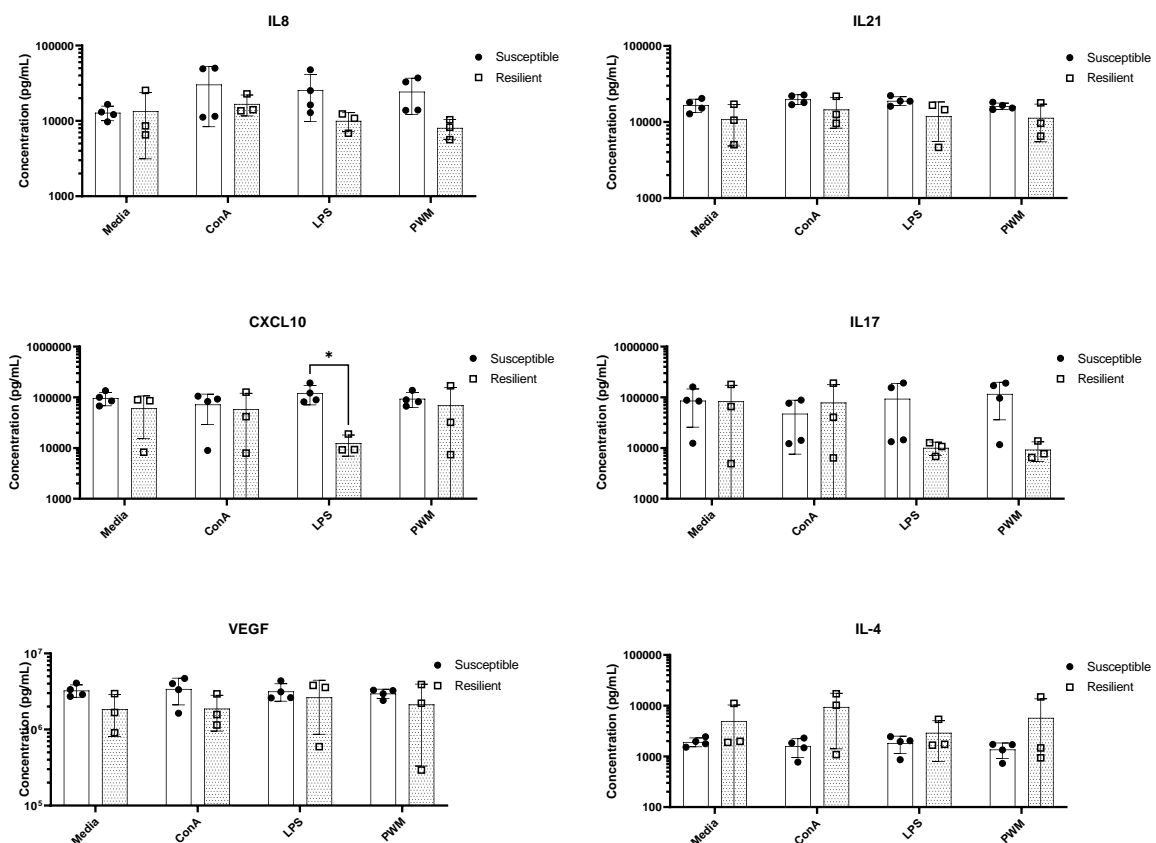
Figure 4.2.1. Preliminary screening of cytokines in whole blood cultures incubated with nonspecific stimuli in sheep prior to exposure to transportation and pathogen stress. Samples were collected while the sheep were still at their farm of origin, prior to transportation and start of the experimental trial where animals were exposed to an infectious dose of *Salmonella*. Animals were grouped based on disease outcome at the end of the trial. n=4 Susceptible; n=3 Resilient.

4.2.2.2   Cytokine responses after infectious challenge

Samples from sheep subjected to multiple pathogen challenge (section 3.1.1) were also screened by multiple cytokine array. Stimulated blood samples from 4 animals (n=2 per group) collected 3 weeks after challenge with Hc and 2 weeks after challenge with MAP (i.e. 3 weeks post primary challenge, wppc). Animals were initially grouped based on change in weight during the first 6 months of the trial as disease outcome was not known at the time the array samples were processed. An average daily gain less than 10 g was defined as Low and greater than 20 g was defined as High. Five of the twenty cytokines were excluded from further analysis as they did not meet assay criteria.

Weight group was a significant effect (p<0.05, two-way ANOVA) for the following cytokines: decorin, AIF, IFNα, IL-1α, IL-1β and TNFα (Appendix 8.1). Decorin and TNFα stand out as having the potential to discriminate between animals that were able to gain weight in the presence of multiple burden (Fig. 4.2.2). For both cytokines, the response was higher in the Low weight group compared to the High group. For decorin, significant differences were noted in the unstimulated blood samples and those cultured in the presence of LPS. Decorin is an extracellular matrix protein which acts as a ligand of various cytokines and growth factors and can affect pro-inflammatory processes (Zhang et al., 2018).The pro-inflammatory cytokine TNFα (Idriss & Naismith, 2000) was significantly different between the two groups in Con A, PWM or PPDa stimulated samples. PPDa (purified protein derivative from *M. avium*) was used as an indicator of exposure to environmental mycobacteria.
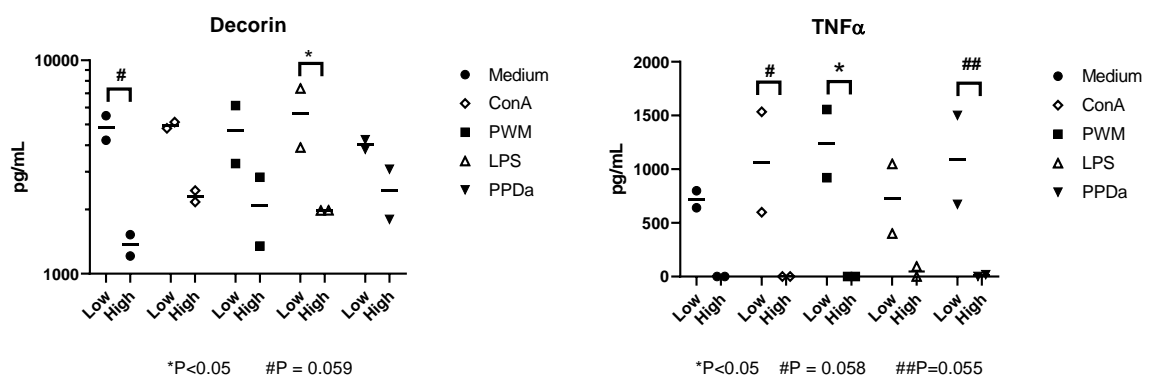
Figure 4.2.2. Preliminary screening screening of cytokines in whole blood cultures incubated with nonspecific stimuli in sheep subjected to multiple pathogen challenge. Blood samples from sheep challenged with Hc and MAP collected at 3 weeks post primary challenge were cultured in the presence of a variety of stimuli which target cellular activation. Cytokine secretion was assessed using a protein array. n=2 per group. Low weight gain = less than 10 g/day in 6 months. High weight gain = greater than 20 g/day in 6 months

Overall trends from the array data (**Appendix 8.1**) indicated that other cytokines such as CXCL9, IL-17 and CXCL10 were worth pursuing further.

Stimulated blood samples from the same co-challenge trial were also assessed based on disease outcome (**Appendix 8.1**) but did not provide additional information towards the selection of cytokines for validation by ELISA.

4.2.2.3 Cytokine responses at induction to a commercial feedlot

A subset of ten (n=5 per group) cattle from a commercial feedlot (section 3.1.6) were retrospectively selected for this initial screening based on change in weight during the period under study. Cattle with weight gain of less than 20% of weight at induction to the feedlot were classified as Low while animals with weight gain greater than 35% were classified as High. Overall, there were no statistically significant differences between the two groups for any of the compounds used to stimulate whole blood (**Appendix 8.1**).

Where there tended to be differences between groups, cytokine induction was greater in the lower weight gain group (Fig. 4.2.3). The lymphocyte stimulants Con A and PWM and the mix of mycobacterial antigens in PPDa tended to induce Monocyte Chemoattractant Protein 1 (MCP-1) to a greater level in the low weight gain group compared to the high weight gain cattle. LPS and PPDa tended to induce vascular endothelial growth factor (VEGF) in the low weight group animals but not in the higher weight group. IFNα was higher in the low weight group for all stimuli as well as in the unstimulated blood samples. Con A and LPS induced Monocyte Inflammatory Protein 1b (MIP1β) in the low weight group and this was apparent in the unstimulated (medium alone) samples too. MCP-1 directs the movement of monocytes and macrophages to sites of active inflammation. MIP1β is also a monocyte chemoattractant and IFNα is involved in innate immune responses.

These responses suggest that cattle that are not gaining weight at the same rate as others in the cohort have more potent immune responses. Elevated cytokines in the unstimulated samples provides further support to this assumption.
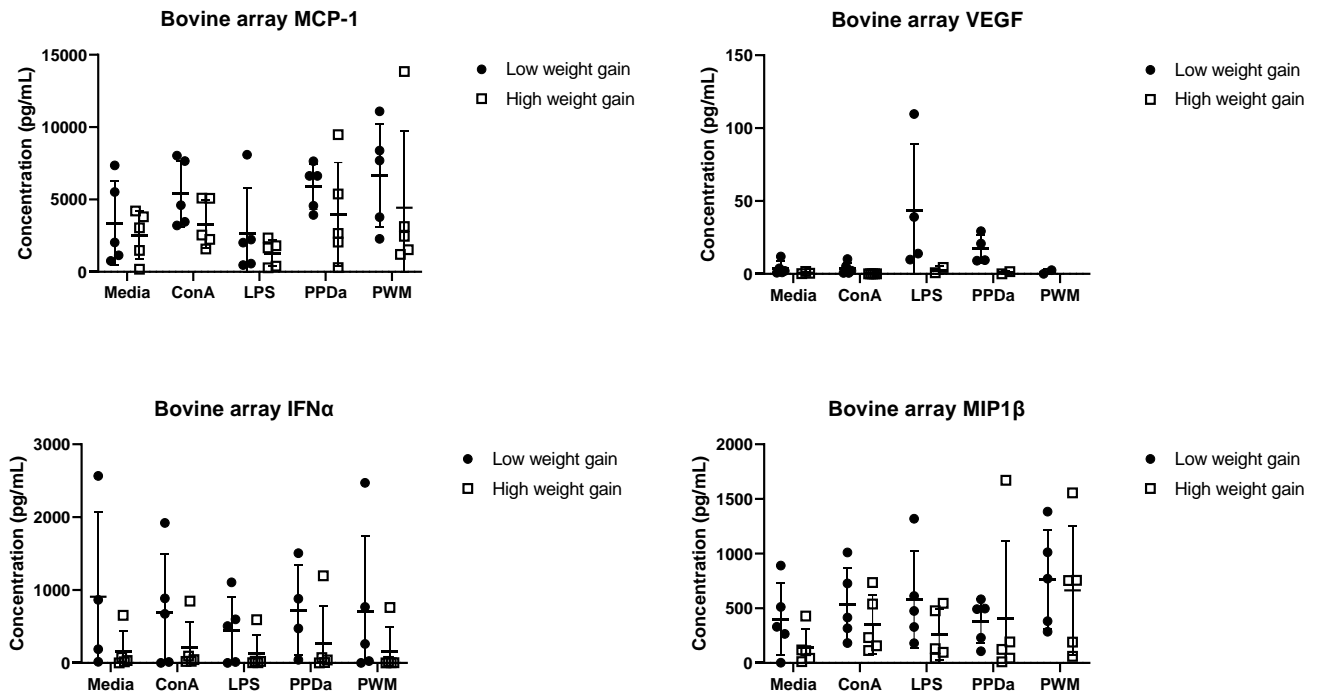
Figure 4.2.3. Preliminary screening for cytokines stimulated by nonspecific stimuli in feedlot cattle at induction. Blood samples from feedlot cattle were collected at induction. Weight gain less than 20% was defined as Low and greater than 35% as High. n=5 per group.

4.2.3 ELISA validation of putative protein immune biomarkers

Based on the preliminary assessment of cytokine array results and the availability of reagents to detect ovine and bovine cytokines, induction of the following cytokines was assessed by ELISA: CXCL-10, IFNα, IL-8, IL-17, IL-1β and IFNγ. While Decorin was identified as a potential biomarker in the initially screening by array technology, we were unable to include it in the validation studies as reagents for an ELISA is not currently available. Statistical analyses were carried out only where there were sufficient animals per group.

#### 4.2.3.1 Cytokine responses following multiple pathogen challenge

Samples were from the co-challenge model in sheep (Section 3.1.1) taken at pre-challenge, 3 and 14 wppc. Two pathogen specific antigens, Hc antigen and 316v (MAP) were also included as whole blood stimulants to assess the pathogen-specific response. While control animals were held on a separate paddock to remain as a nonexposed group, they were unknowingly exposed to Hc contaminated pasture; these sheep were also categorised as susceptible and resilent groups based on the FWEC.

None of the cytokines differentiated between disease outcome groups regardless of the nonspecific compound used to stimulate immune cells in whole blood following in vivo infectious challenge. Overall, the only observed differences between the co-challenged susceptible and resilient groups was in the antigen-specific IFNα response (Fig 4.2.4) where the response in resilient animals tended to be lower to PWM as well as the pathogen specific antigens. This type-I interferon is an important regulator or cellular communication and signal transduction and has both pro- and anti-inflammatory effects (Tilg et al. 1996).
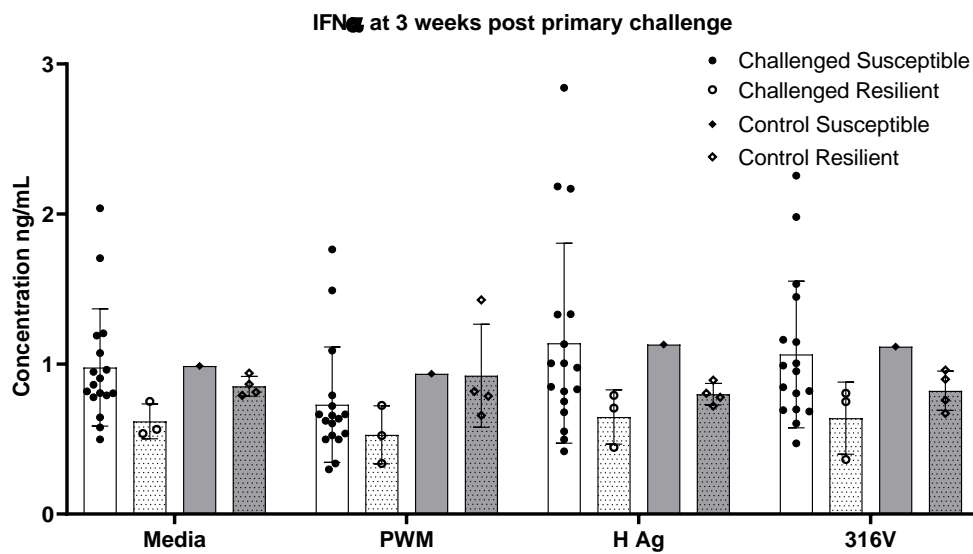
Figure 4.2.4. Interferon alpha (IFNα) responses in sheep co-challenged with Hc and MAP

Prior to pathogen exposure, PWM significantly induced IFNg in all animals compared to unstimulated blood (Fig. 4.2.5). At 3 wppc, this response was signficantly lower in the co-challenged susceptible compared to the resilient sheep in the same group. When comparing the susceptible groups, the co-challenged had a lower response to the 'controls'.
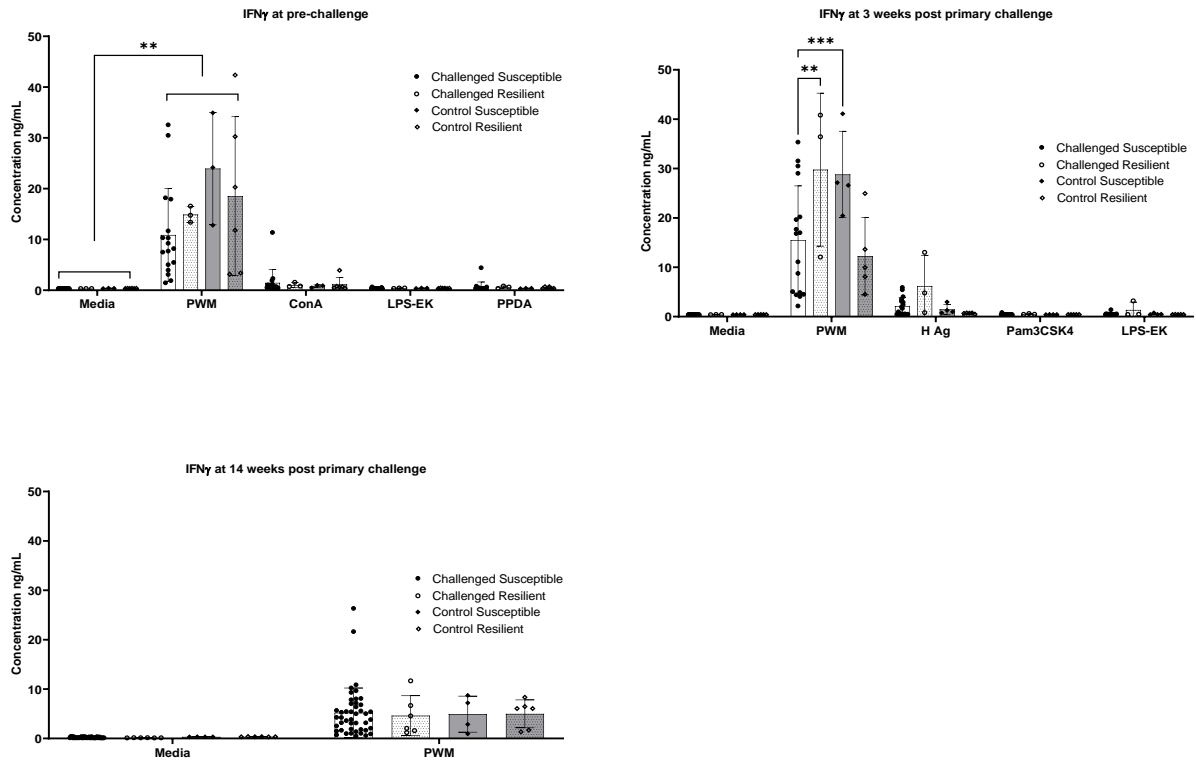
Figure 4.2.5. Interferon gamma (IFNγ) responses in sheep co-challenged with Hc and MAP

**p<0.01 ***p<0.001

Similar responses were observed with IL-8 and IL-1β (Fig. 4.2.6). IL-8 is a chemotactic cytokine which mainly targets neutrophils and lymphocytes to migrate to the site of infection (Sharma, Lall & Kishore, 2015). It plays an important role in inflammation due to its ability to activate neutrophils and initiate innate immune mechanisms as first-line defence pathways against pathogens. IL-1β is a key pro-inflammatory mediator secreted in response to infection or injury (Lopez-Castejon & Brough, 2011). Pathogen associated molecular patterns (PAMPs) induce production of the precursor, pro-IL-1β. Subsequently a secondary signal is required to convert this to its functional form. At 3 wpcc, the IL-8 response tended to be higher in the challenged animals with or without antigen stimulation compared to the control group (Fig. 4.2.6). This early response is likely due to pathogen exposure. IL-1β in unstimulated samples was low, indicative of the tight regulation of this potent cytokine to prevent excessive tissue damage. By 14 wpcc, the PWM-induced IL-1α response in the challenged animals was significantly lower than the controls. Similarly, IL-8 tended to be lower in the challenged animals (Fig. 4.2.6) at this later time point.
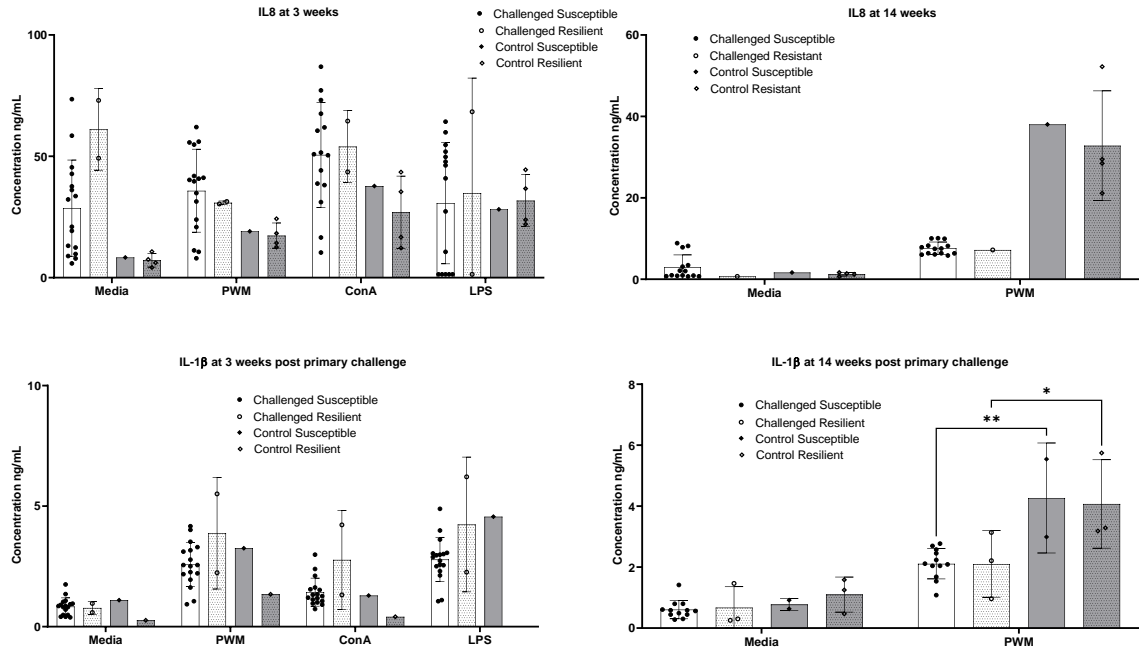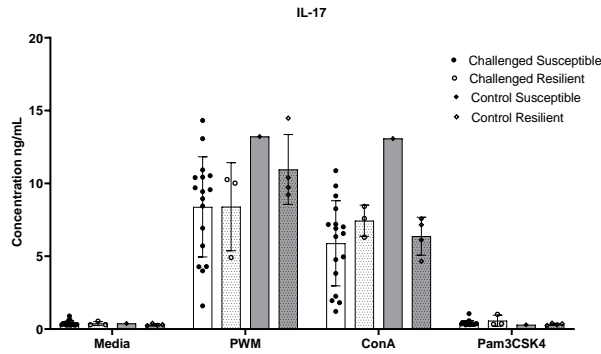
Figure 4.2.6. IL-1β and IL-8 responses in sheep co-challenged with Hc and MAP *p<0.05 **p<0.01

The cytokine CXCL10 is involved in Th1 mediated adaptive immune responses (Metzemaekers et al., 2018). Due to its functional properties it was previously known as IFNγ-inducible protein 10 (IP10). Induction of the CXCL10 response by the generic PWM stimulus was significantly higher in the co-challenged sheep compared to the controls at 14 wppc (4.2.7). For the pathogen specific antigens (Hc and MAP) there were significant differences between co-challenged susceptible and the control susceptible (Hc natural exposure) groups. This is likely a reflection of differences in infectious dose and/or the multiple vs single nature of the infectious burden. At the earlier time point of 3 wppc the PWM-induced CXCL10 response was significantly different between the co-challenged susceptible and control resilient group.
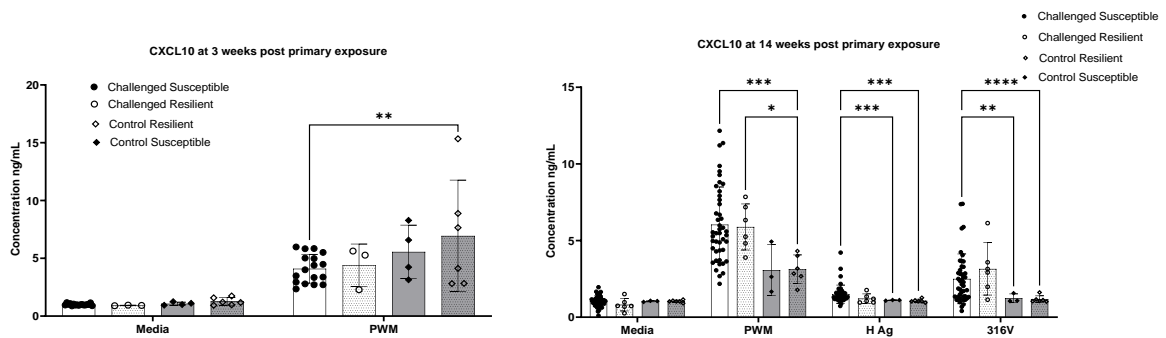
Figure 4.2.7. CXCL10 responses in sheep co-challenged with Hc and MAP H Ag= Haemonchus antigen; 316v=MAP antigen *p<0.05 **p<0.01 ***p<0.001

The IL-17 response to both PWM and Con A tended to be lower in challenged animals at 3 wppc (Fig 4.2.8). IL-17 is produced by the T helper lymphocyte subset Th17 and plays an important role in driving both inflammation and tissue repair (Li et al., 2019). It can act synergistically with other factors such as IFNγ and LPS.

Figure 4.2.8. IL-17 responses in sheep co-challenged with Hc and MAP

### 4.2.3.2   Cytokine responses following single pathogen challenge

Sheep

The pattern of select cytokine responses was validated in sheep before and after challenge with a single bacterial pathogen (Salmonella). Samples were collected after sheep had been transported to the experimental trial site prior to pathogen exposure as well as 7 days after exposure when infectious burden was high (Fig. 4.2.9). Sheep were categorised into disease susceptible and resilient groups at the end of the trial. Four animals per group were retrospectively selected for cytokine analysis. Prior to pathogen exposure, there were no significant differences between disease outcome groups (**Appendix 8.1**).

At 7 days post exposure, the CXCL10 response to all stimuli tended to be higher in the resilient animals; with the poly(IC)-induced response significantly elevated in this groups (Fig. 4.2.9). This compound is a TLR3 antagonist which simulates the binding of double stranded DNA is generally associated with IFNb secretion but is also known to induce CXCL10 (Taima et al., 2006).

The IL-1β response to PWM at 7 days post exposure was significantly higher in the susceptible animals compared to the resilient group (Fig. 4.2.9). While the LPS response was similar it was not significantly different.

The PWM-induced IL-8 response was significantly lower in the resilient animals.

All stimulants assessed also tended to induce higher IFNα responses in resilient animals compared to the susceptible (**Appendix 8.1**). PWM and Con A induced IL-17 secretion but there was no difference between the two disease outcome groups (**Appendix 8.1**). IFNγ response to PWM was higher in the resilient group but was not statistically significant (**Appendix 8.1**).

CXCL10

IL1β
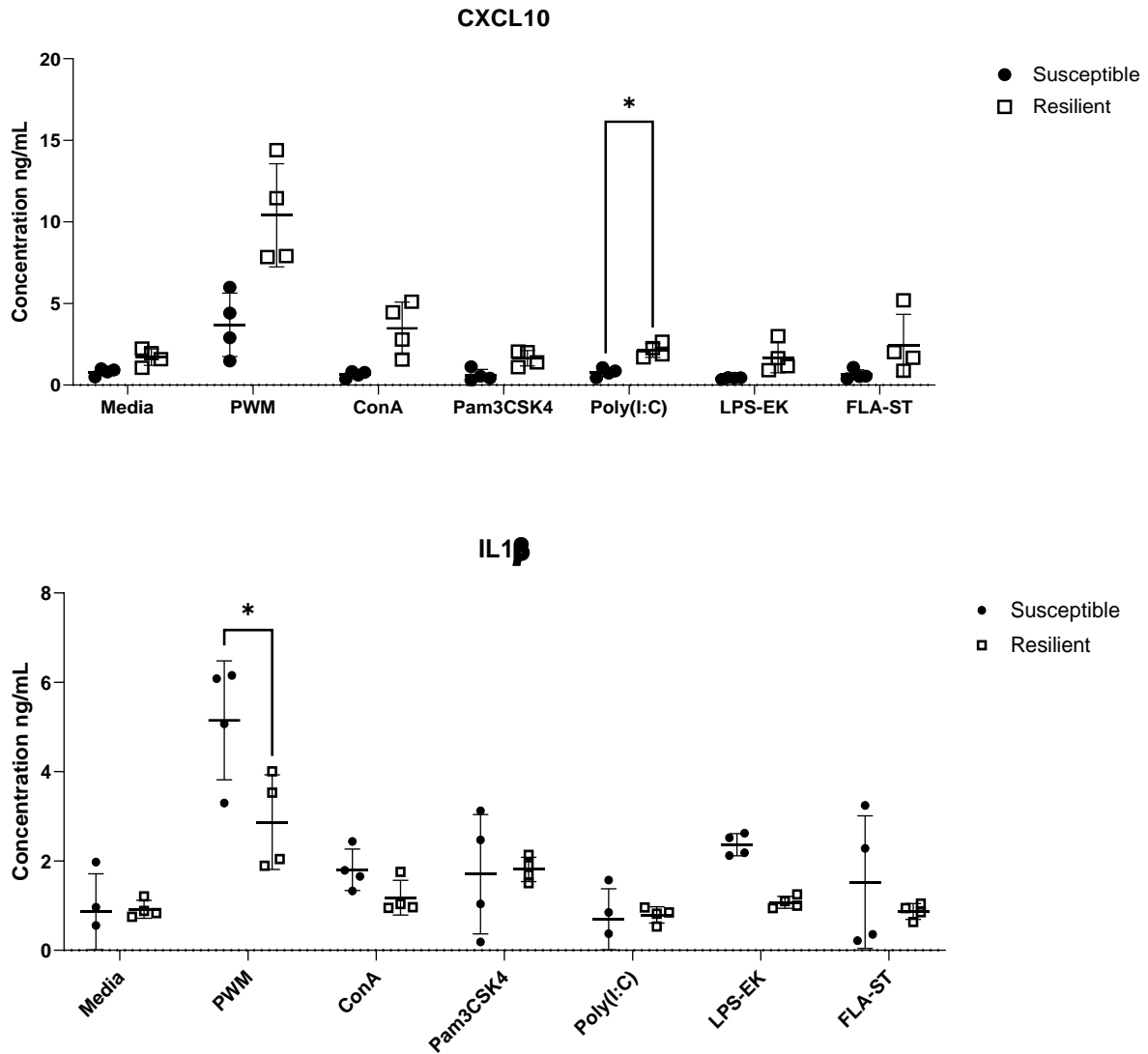
Figure. 4.2.9. Cytokines stimulated by nonspecific stimuli in sheep based on disease susceptibility in the presence of a single pathogen challenge. Blood samples from sheep collected 7 days post Salmonella exposure were cultured in the presence of a variety of stimuli which target cellular activation. Cytokine secretion was detected by ELISA. Animals were categorised based on susceptibility to disease (n=4 per group).
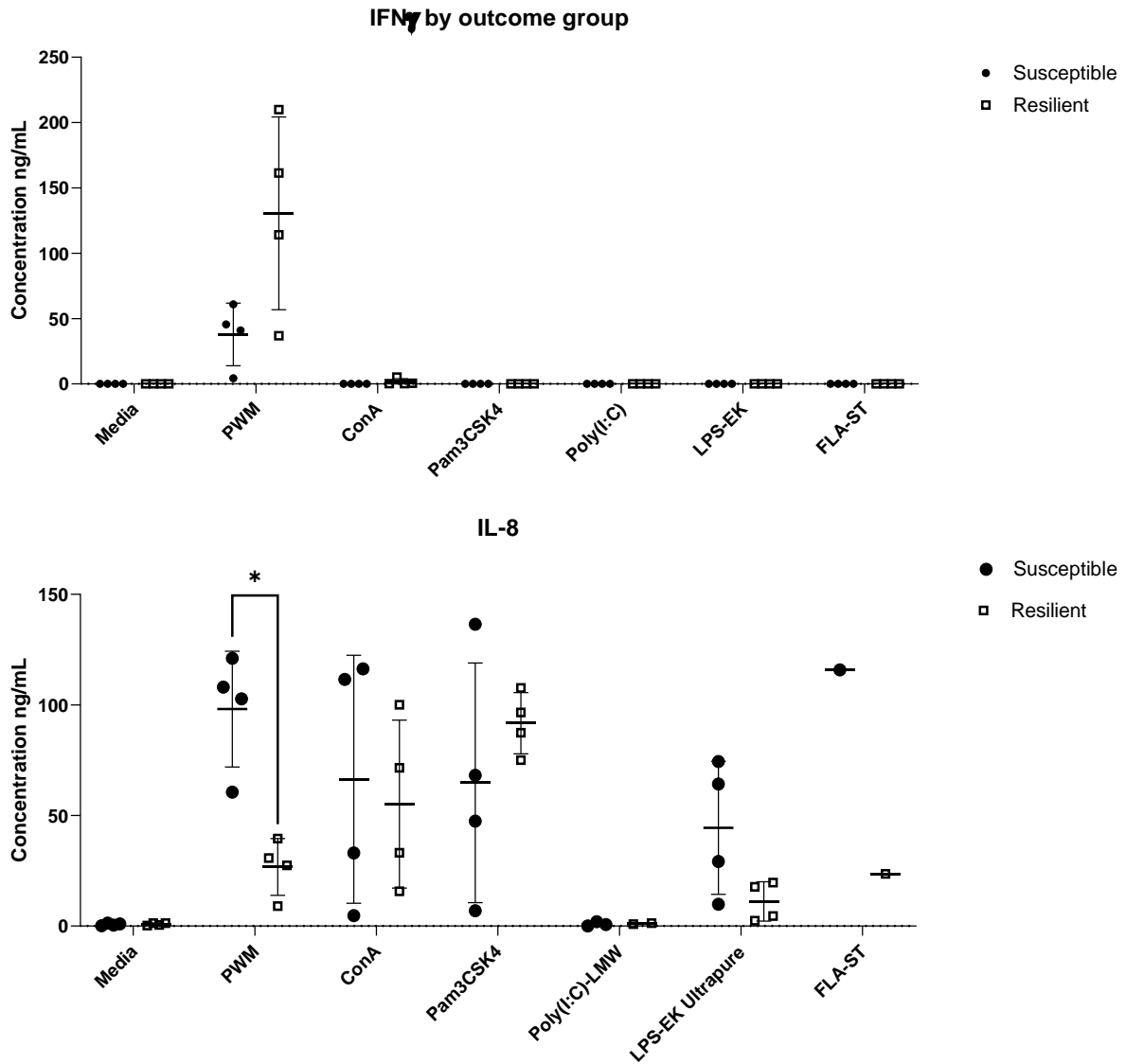
Figure. 4.2.9. cont. Cytokines stimulated by nonspecific stimuli in sheep based on disease susceptibility in the presence of a single pathogen challenge. Blood samples from sheep collected 7 days post Salmonella exposure were cultured in the presence of a variety of stimuli which target cellular activation. Cytokine secretion was detected by ELISA. Animals were categorised based on susceptibility to disease (n=4 per group).

Cattle

Archived whole blood culture supernatants from a previous cattle trial (Section 3.1.7) were used to assess variation in the PWM-induced IFNg response in relation to disease outcome and weight gain (Fig. 4.2.10). Resilient animals had no detectable MAP granuloma or MAP DNA in multiple faecal samples (n=18) while susceptible animals were positive for both these criteria (n=2). Weight groups were defined as Low for animals where percentage weight gain was 118% or lower (n=7), Mid for animals between 119-130% (n=7) and High for animals with greater than 130% gain (n=6) during the period under assessment.

While there were no statistically significant differences between the two disease outcome groups, higher weight gain at 56 wppc (MAP) was associated a significantly lower PWM-induced IFNγ response compared to the lower weight gain groups (Fig 4.2.10).
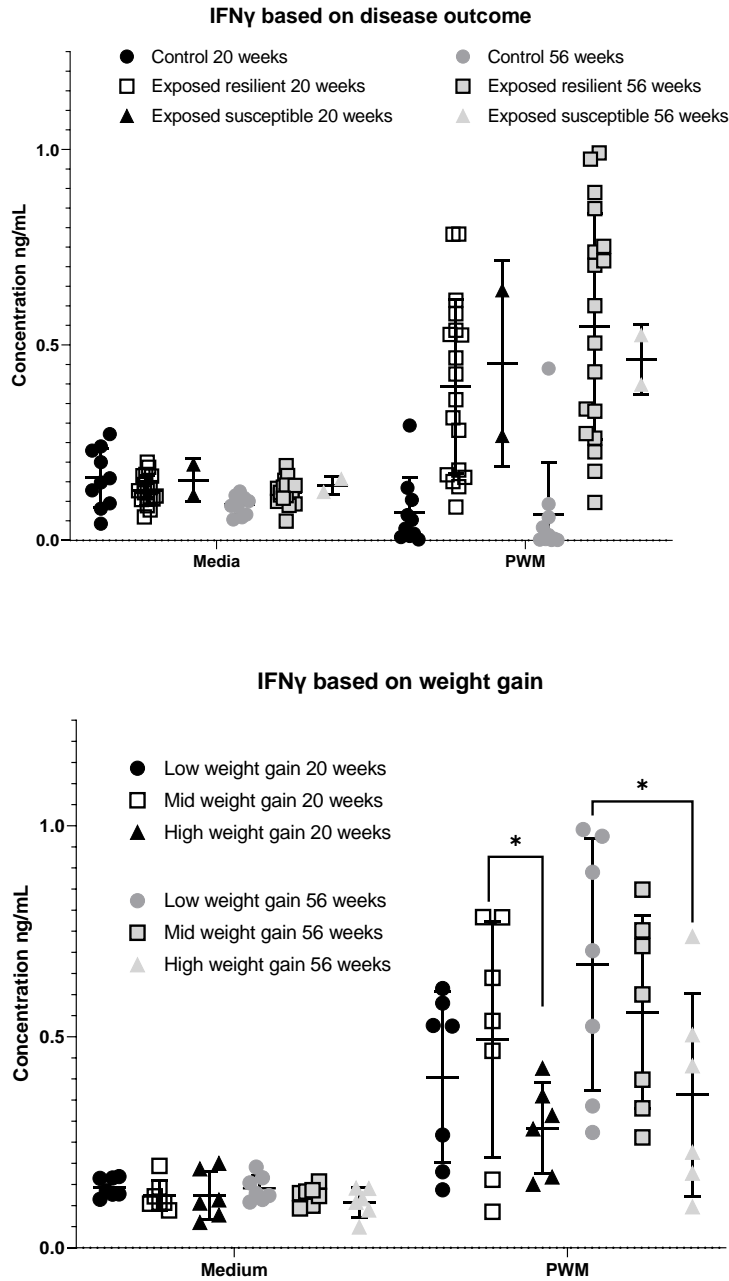

Figure 4.2.10. IFNγ response in relation to disease outcome in cattle and weight gain subjected to pathogen (MAP) exposure

4.2.3.3   Cytokine responses in animals during normal husbandry practices

Sheep

For the sheep model, blood was collected from 4 month old lambs at a time point considered to be relevant to stress accociated with weaning, vaccination and ear tagging from a commercial farm. Animals were categorised based on percentage weight gain during the 210 day period under study. Animals with weight gain during this period of 50% or less were categorised as Low and those with weight gain of 81% or greater were categorised as High. Ten sheep from within these categories were retrospectively selected for assessment of cytokine induction in the presence of non-specific stimuli (Fig 4.2.11). For most of the cytokines, the strongest inducer was PWM. The elevated IFNγ response to PPDa indicates that these sheep had exposure to *M. avium* species. PAM3CYSK4, LPS and PPDa all induce an IL-8 response. There were no significant differences between the low and high weight gain groups for any of the cytokines.
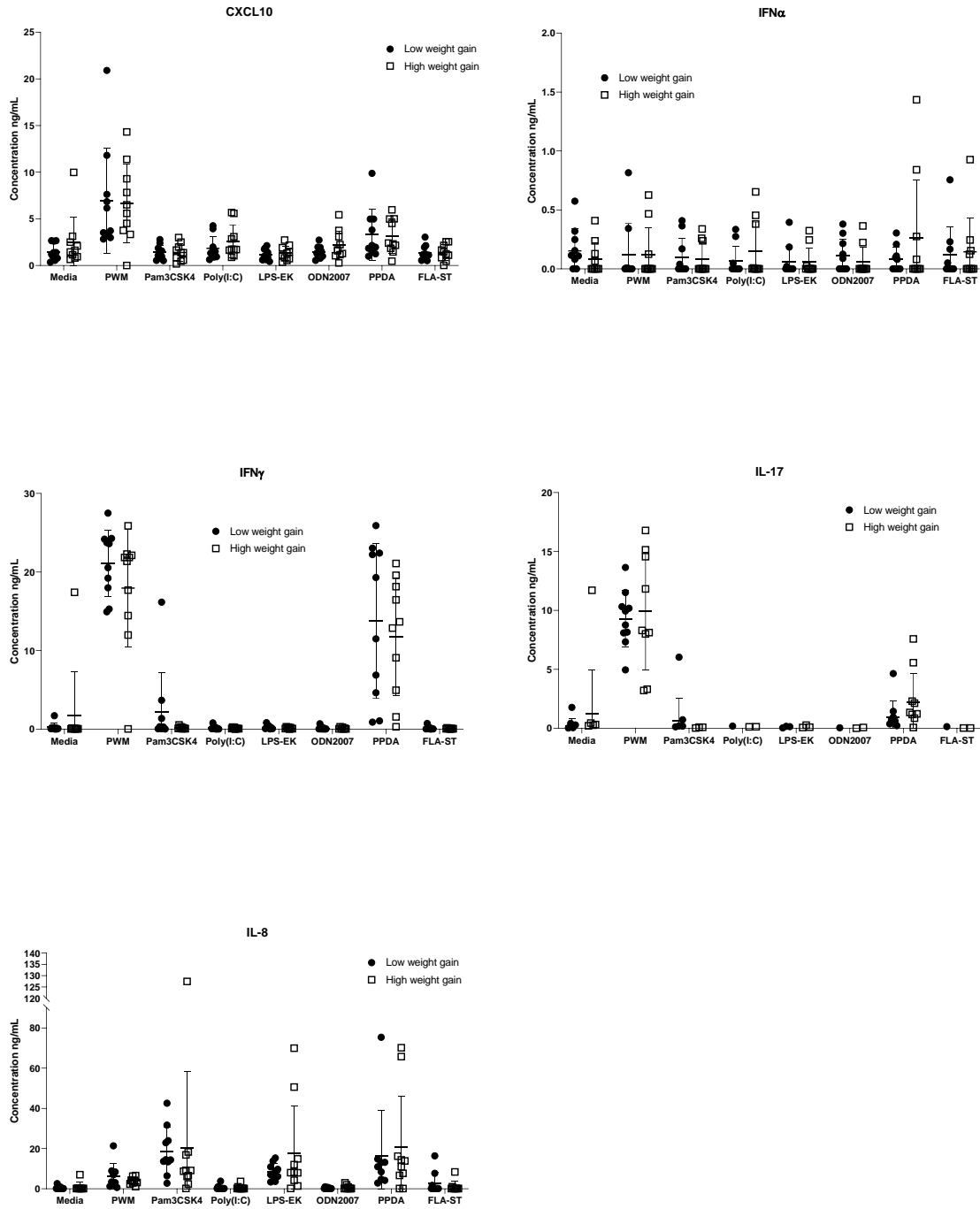
Figure 4.2.11. Cytokines stimulated by nonspecific stimuli in sheep based on weight gain in sheep under normal husbandry practices on-farm. Blood samples from sheep collected at 4 months of age were cultured in the presence of a variety of stimuli which target cellular activation. Cytokine secretion was detected by ELISA. Animals were categorised based on percentage weight gain during a 210 day period (n=10 per group). Low = weight gain at or below 50% of starting weight; High = weight gain at 81% or greater or starting weight

Cattle

For the cattle model, four cytokines IFNα, IFNγ, CXCL10 and IL-1β, were selected for further validation of cytokine responses in relation to immune fitness of cattle. These were selected based on the availability of bovine cytokine ELISAs and general trends observed from the initial screening using the 30-cytokine array. Animals from the same feedlot cohort were selected based on weight gain during the period under assessment. The Low group gained less than 25% of weight at induction while the High group gained more than 25% of weight at induction to the feedlot (n=16 per group).

There were no statistically significant differences in the stimulant-induced cytokine responses between the two weight gain groups (Fig. 4.2.12). In contrast to the cytokine array, the overall tendency appears to be for higher induction of cytokine responses in the high weight gain group; PWM tended to induce higher IFNγ and CXCL10 responses in this group. IFNα in whole blood culture supernatants were not detectable by ELISA; this could be due to the sensitivity of this assay.
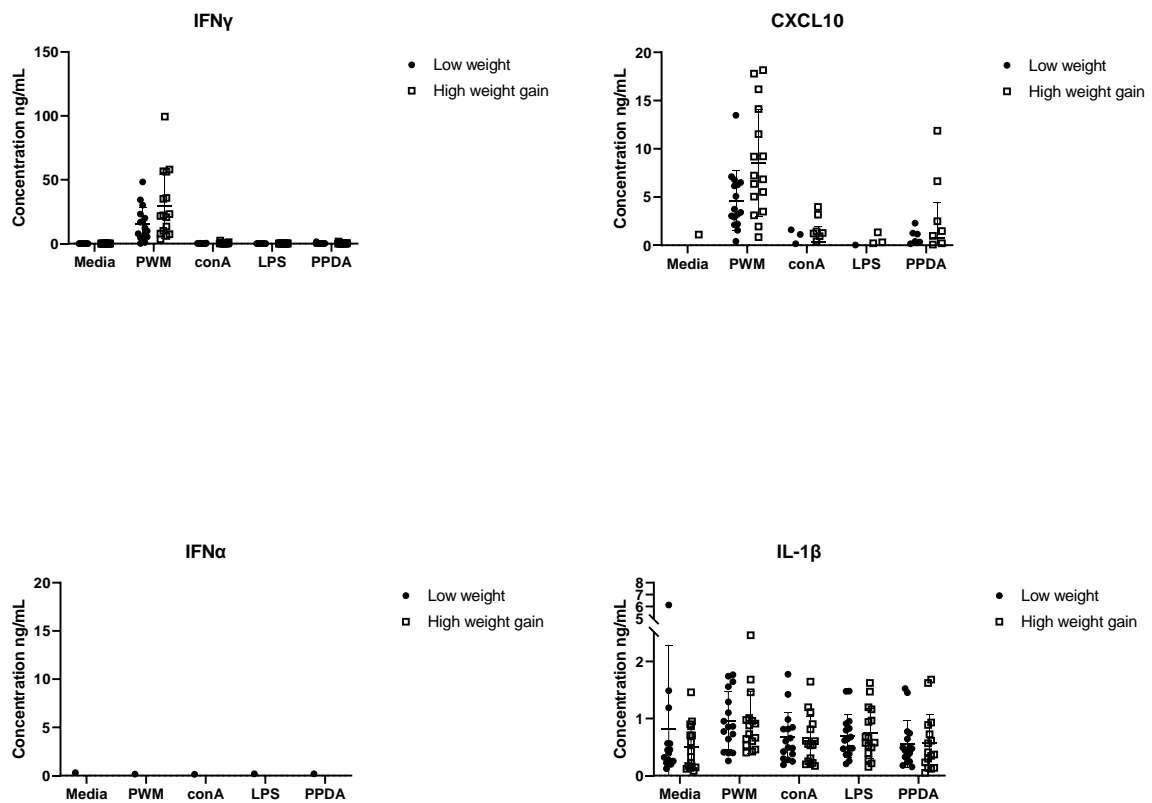
Figure 4.2.12. Cytokines stimulated by nonspecific stimuli in feedlot cattle based on weight gain in sheep under normal husbandry practices on-farm

We also assessed these cytokine responses based on animals' MSA index but did not find any differences between groups (Fig. 4.2.13).
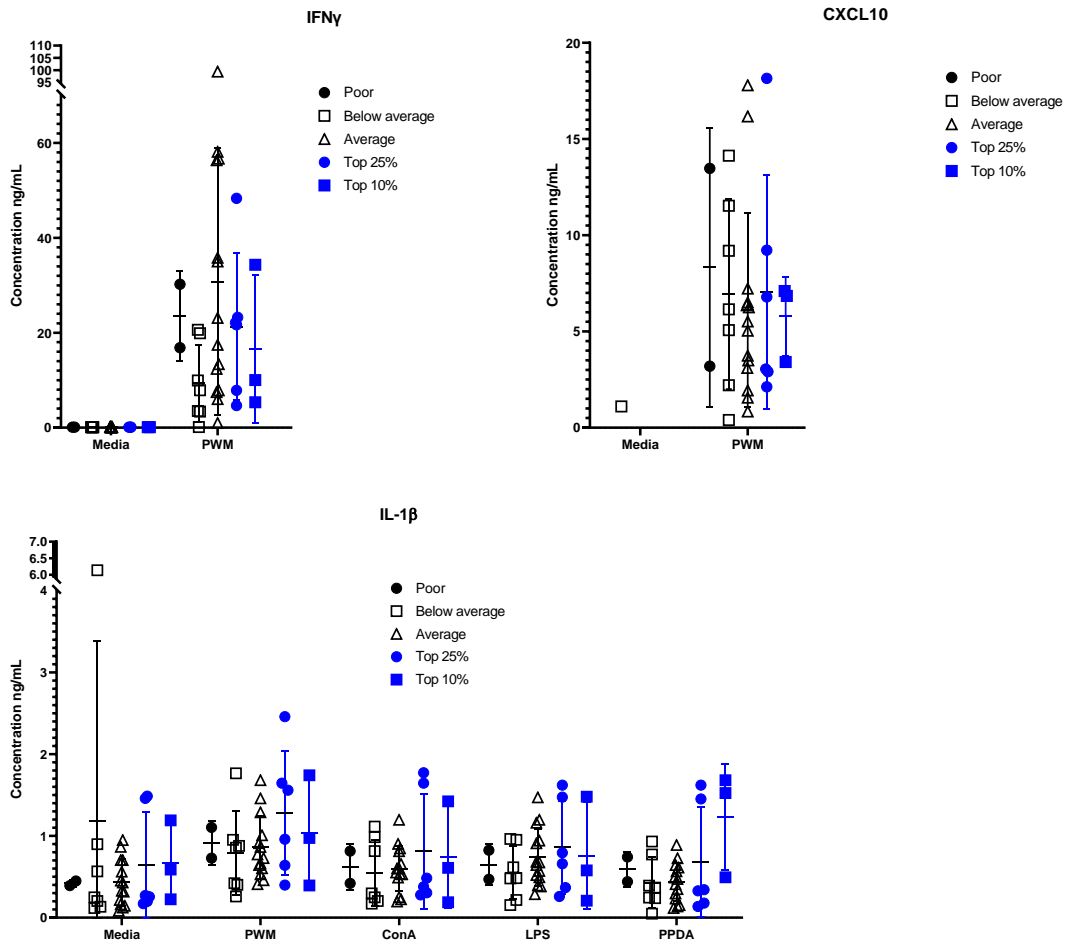
Figure 4.2.13. Cytokines stimulated by nonspecific stimuli in feedlot cattle based on weight gain in sheep under normal husbandry practices on-farm

Overall cytokine outcomes are summarised in Table 4.2.2

Table 4.2.2. Overall summary of cytokine patterns differentiating between disease or weight outcomes in sheep and cattle

| Cytokine	Stimulant	Stress	Time of sample collection	Outcome groups	Cytokine Response
CXCL10	LPS	Single pathogen	Prior to stress	Susceptible vs resilient	Higher in susceptible
CXCL10	PWM, pathogen specific antigens	Single or multiple pathogen	14 wppc	Susceptible vs resilient	Higher in susceptible
CXCL10	Poly(I:C)	Single pathogen	7 days post challenge	Susceptible vs resilient	Lower in susceptible
Decorin	Unstimulated and LPS	Multiple pathogen	3 wppc	Low vs high weight gain	Higher in low weight gain
TNFα	Con A, PWM, LPS	Multiple pathogen	3 wppc	Low vs high weight gain	Higher in low weight gain
IFNγ	PWM	Multiple pathogen	3 wppc	Susceptible vs resilient	Higher in susceptible
IFNγ	PWM	Single pathogen	56 wppc	Weight gain	Lower in high weight gain
IL-1β	PWM	Single or multiple pathogen	14 wppc	Multiple pathogen susceptible vs single pathogen susceptible	Higher in single pathogen susceptible
IL-1β	PWM	Single pathogen	7 days post challenge	Susceptible vs resilient	Higher in susceptible
IL-8	PWM	Single pathogen	7 days post challenge	Susceptible vs resilient	Higher in susceptible

4.2.4   Cortisol

4.2.4.1   Faecal and wool cortisol levels in control and pathogen-exposed sheep

The faecal cortisol metabolite levels were variable over time and between animals in the co-challenge trial, as shown in Fig. 4.2.14. The exposed animals showed an increase in average FCM levels at 7 and 16 weeks post-challenge compared to 5 weeks; this was significantly higher at both timepoints than the control animals. However, at the first sampling timepoint (5 weeks post inoculation), several animals (animal key list 95-100) from the control group exhibited elevated FCM levels (Fig. 4.2.14 B), leading to a significantly higher FCM for control animals over exposed at this timepoint.
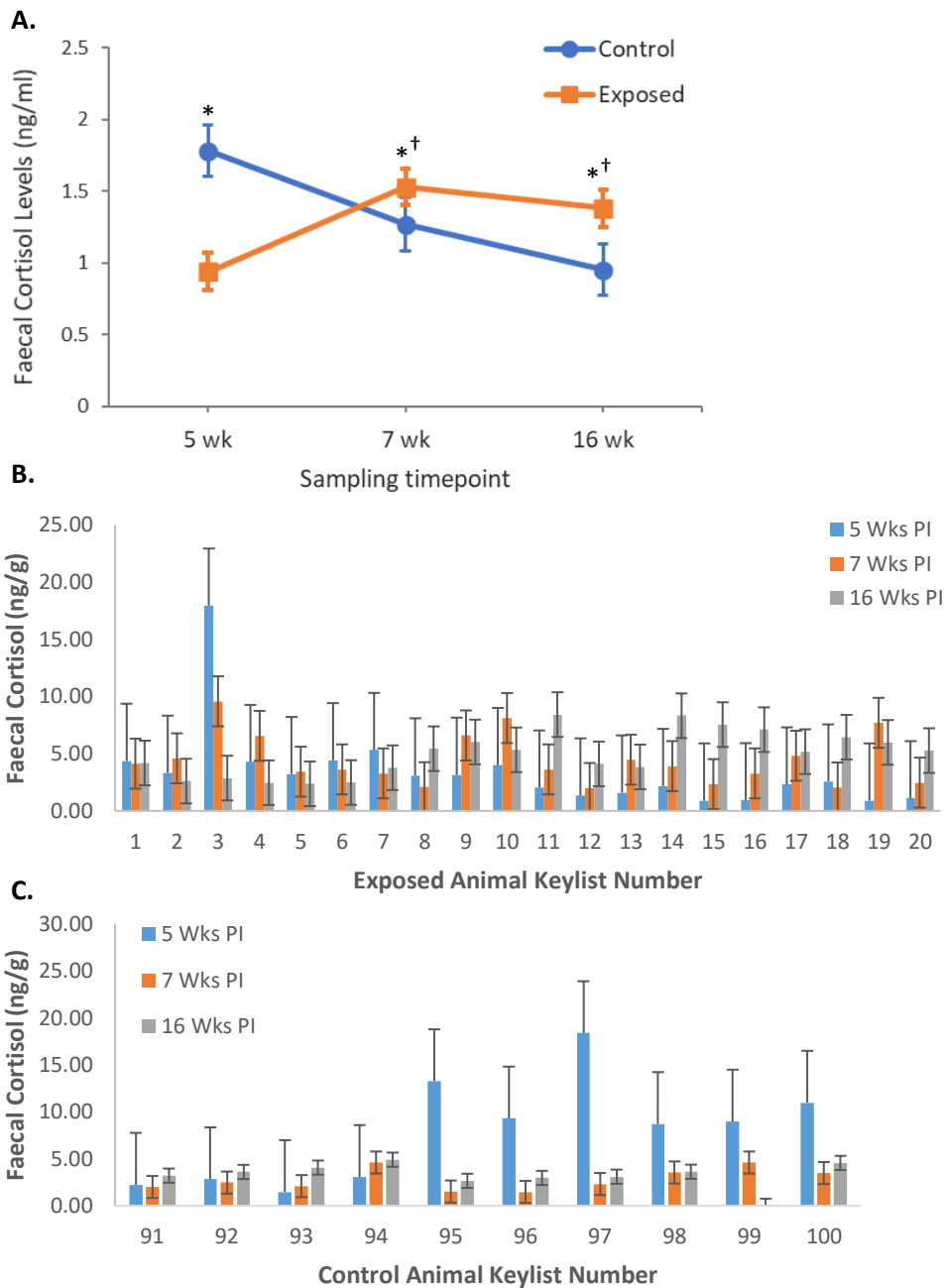
Figure 4.2.14. Faecal cortisol levels (ng/g) in sheep from the co-challenge trial over three sampling timepoints (5, 7 and 16 weeks post-challenge). A. Average cortisol levels (Log transformed ng/ml) and standard errors from the restricted maximum likelihood linear mixed model (REML) analysis comparing exposed versus control animals at 5, 7 and 16 weeks (wk). * Cortisol levels in the exposed group were significantly different ($p<0.001$) to the control group at all timepoints; at 5 weeks post challenge the control group exhibited a significantly higher average cortisol levels compared to animals in the exposed group, while at 7 and 16 weeks the exposed group had higher cortisol levels than the control group. † Cortisol levels detected in the faeces of animals within the exposed group were significantly increased ($p<0.001$) at 7 and 16 weeks compared to the 5 week timepoint for this group. Average cortisol levels detected in the faeces in animals within the control group significantly decreased from 5 to 7 weeks and from 7 to 16 weeks. Panel B and C. Faecal cortisol levels (ng/g) in the individual exposed (B) or control (C) animals.

There was a trend for the wool cortisol levels in the exposed animals to be higher than the animals in the control group, although this difference did not reach significance (p=0.061) (Fig. 4.2.15A). When the faecal and wool cortisol measurements were compared, there was a moderate correlation (r = 0.47 (95% confidence interval (CI) 0.04 to 0.76), p = 0.036) observed between the two measures (Fig. 4.2.15B).
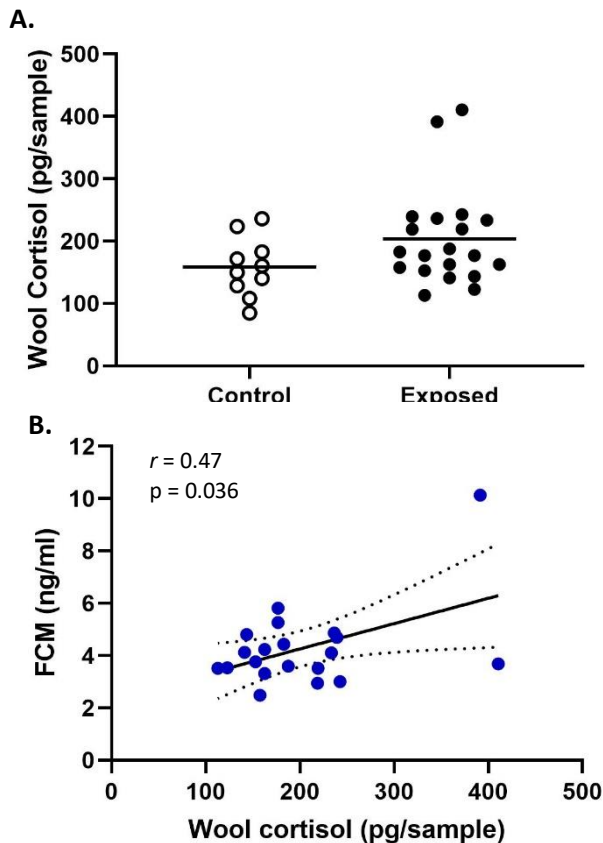
Figure 4.2.15. Scatterplot showing the wool cortisol levels and comparison to faecal cortisol measurements. A. Wool cortisol levels in the control versus the exposed group in the co-challenge trial. B. Correlation between wool cortisol and average FCM across the three timepoints (5, 7 and 16 weeks). The Pearson's correlation r and p value shown on the graph and the regression line (solid) and 95% confidence interval (dotted line) are shown.

The faecal and wool cortisol measures were compared to disease severity outcomes for the sheep in the exposed group (n=20), to determine if these were reliable measures of the physiological stress response with respect to these diseases. There was a moderate correlation (r = 0.51 (95% CI 0.08 to 0.78), p = 0.023) observed between the average FCM across the three timepoints and the AFEC (Fig. 4.2.16 A). There was a similar trend for the wool cortisol and AFEC, though this correlation was weak and not significant (Fig. 4.2.16 C). In terms of disease severity in relation to paratuberculosis disease

outcome, neither the FCM nor wool cortisol measures were correlated with the final disease outcome (Fig 4.2.16 B and D) nor HT-J DNA quantities at the endpoint of the trial (see **Appendix 8.2**).
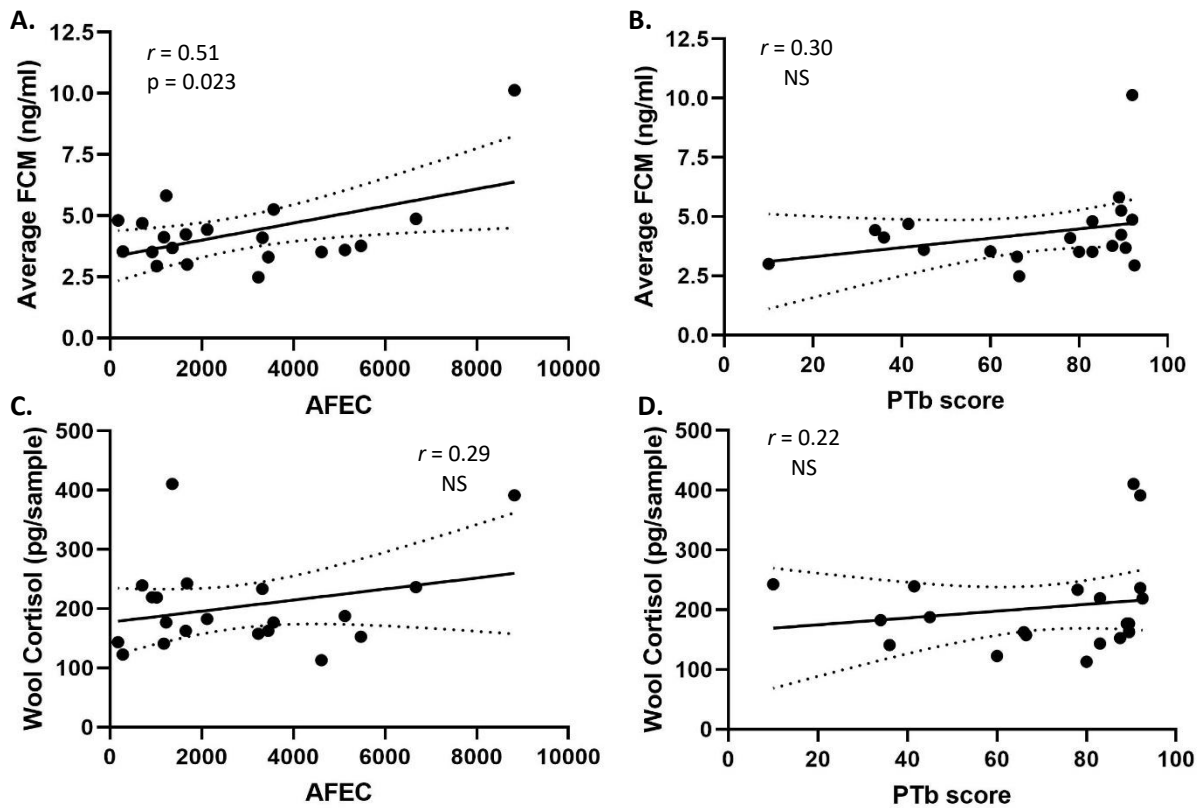
Figure 4.2.16. Scatterplots of the relationship between average faecal cortisol metabolite (FCM) levels or wool cortisol to disease severity measures. Panel A and C. Comparison to average FEC (AFEC). Pearson's correlation *r* and p values are shown on the graphs (NS: p value not significant) and the regression line (solid) and 95% confidence interval (dotted line) are shown. The average faecal cortisol metabolite (FCM) level across the three timepoints (5, 7 and 16 weeks) showed a significant moderate correlation with AFEC. Panel B and D. Comparison to paratuberculosis (PTb) score. Spearman's correlation *r* values are shown on the graphs (NS: p value not significant). The regression line (solid) and 95% confidence interval (dotted line) are shown.

Full results for this study are included in **Appendix 8.2**.

4.2.4.2   Hair cortisol levels in beef cattle

Tail hair cortisol levels from beef cattle from two farms, one of which was bushfire affected and one in a control region of NSW, were compared. Bushfire was seen as a unique source of stress that may be measurable in the tail hair. The hair was cut into four segments, each 3 cm in length. Segment 'a' was the first 3 cm segment of tail hair closest to the animal, whereas segment 'd' was furthest away. The hair was collected between 6 and 9 months after the fires.

There was no significant difference seen in the tail hair cortisol levels of cattle that survived a bushfire versus those from control farms across the sections of hair (Fig. 4.2.17). A high degree of individual animal variability was seen (Fig. 4.2.17 B and C).
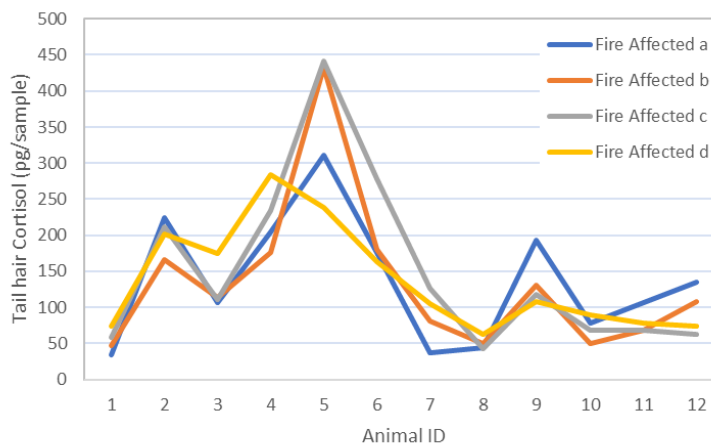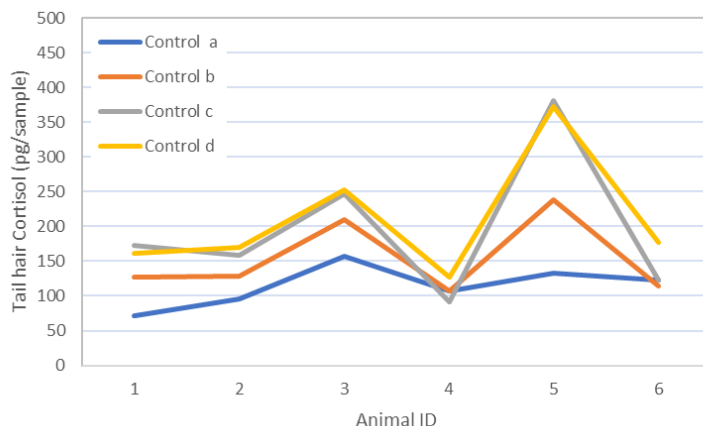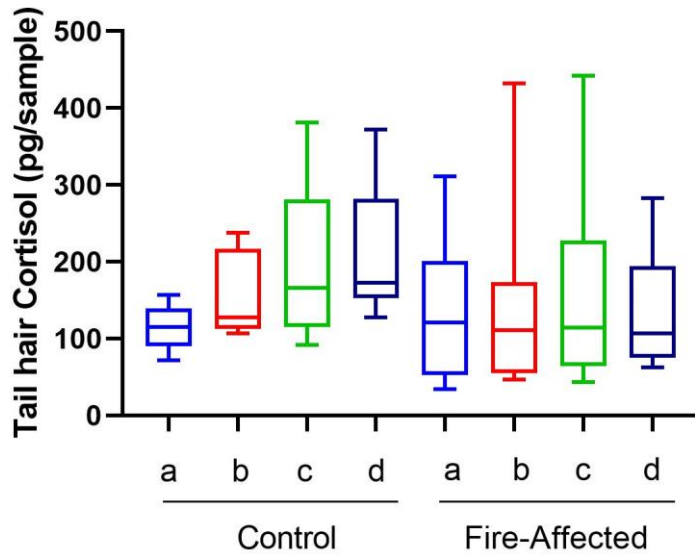
Figure 4.2.17. Hair cortisol levels in beef cattle. Cattle from a farm in a non-fire affected control region (n=6) were compared to a bushfire affected farm (n=12). A. Box and whiskers plot of the average hair cortisol levels in the different regions along the length of the tail hair. Panel B and C. Individual animal results for the different regions along the length of the tail hair for animals from the control (B) and the fire-affected (C) farm.

4.2.5 Trace mineral analysis

Trace minerals were assessed in a subset of animals from the various trials to determine if there was any correlation with immune fitness. There was no association found between disease or production outcomes and the trace mineral levels for animals in any of the trials.

For sheep serum samples, the levels of other minerals varied depending on the animal, with examples shown in Fig. 4.2.18.
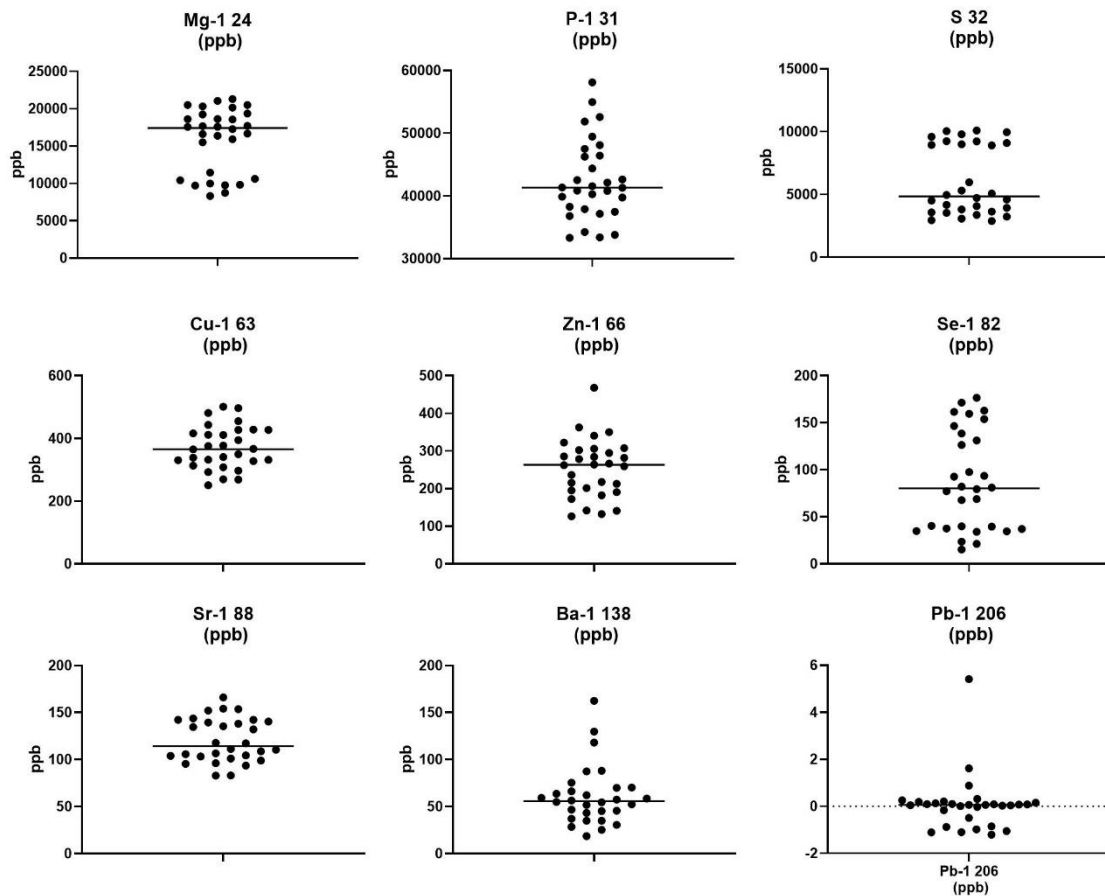
Figure 4.2.18. Trace mineral levels in individual sheep from the co-challenge trial and Salmonella challenge trial. Mg: Magnesium, P: Phosphorus, S: Sulfur, Cu: Copper, Zn: Zinc, Se: Selenium, Sr: Strontium, Ba: Barium, Pb: Lead. Other minerals that were at background levels and are not shown include cobalt, chromium, manganese, nickel, silver and cadmium. Sodium, calcium and potassium were at high levels for all animals and are not shown.

In the co-challenge trial animals that could be characterised as resilient or susceptible to JD were assessed. The results are shown in Fig. 4.2.19 The levels of trace minerals did not show an association to immune fitness.
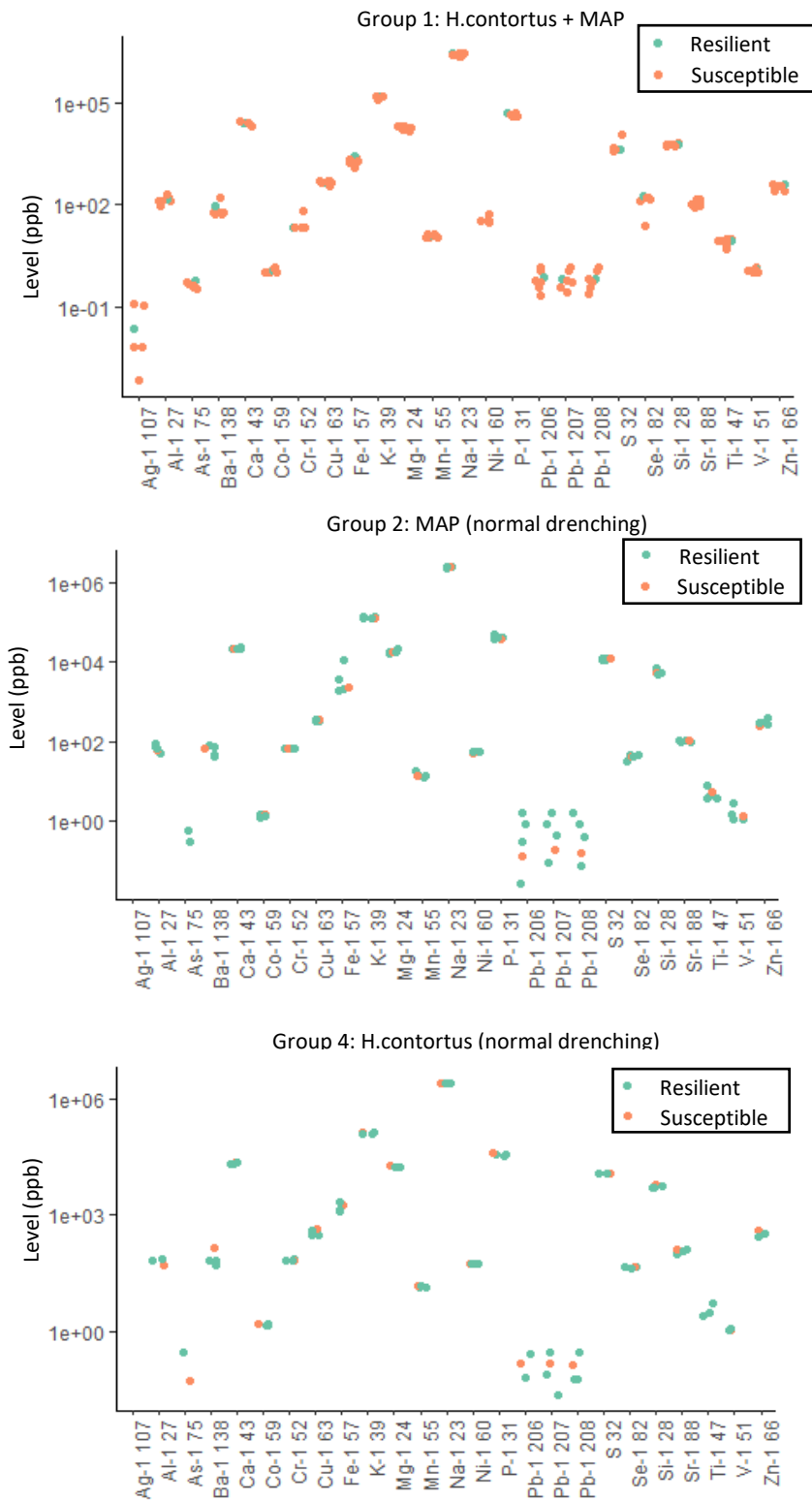
Figure 4.2.19. Analysis of trace mineral levels according to paratuberculosis disease outcome (resistant or susceptible) in the co-challenge trial.

For cattle, trace mineral levels were not strongly correlated with weight gain (Fig. 4.2.20) or MSA Index (Fig. 4.2.21). Only two minerals showed a weak correlation with weight gain proportional to entry weight; Se (selenium) had a weak negative correlation (*r* = -0.34 (95% confidence interval (CI) -

0.5838 to -0.04050); p = 0.028) and Sr (Strontium) had a weak positive correlation (r = 0.34, 95% CI 0.03896 to 0.5828, p = 0.028).
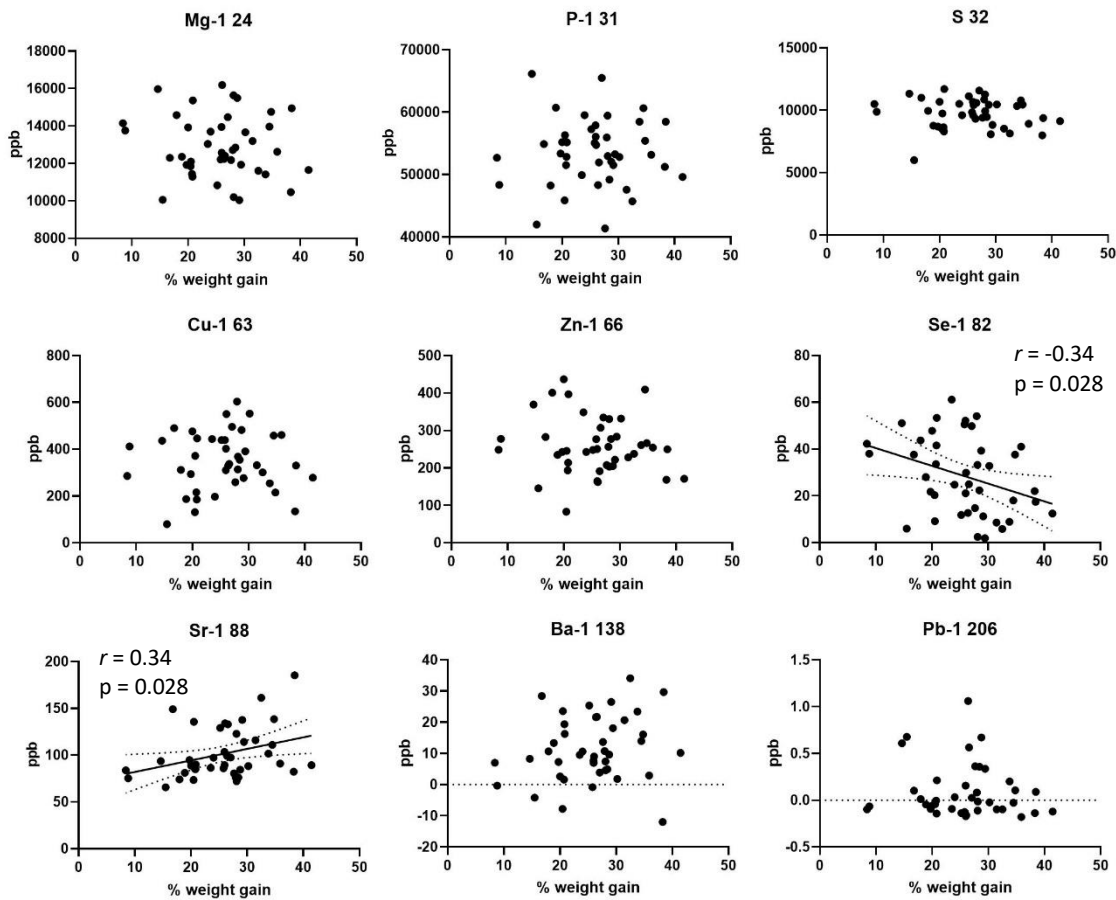
Figure 4.2.20. Scatterplot of trace mineral levels in individual cattle from the feedlot trial versus weight gain proportional to entry weight (%weight gain). The Pearson's correlation and r and p value are included on the graph if there was a significant association, with the regression line (solid) and 95% confidence interval (dotted line) are shown. Mg: Magnesium, P: Phosphorus, S: Sulfur, Cu: Copper, Zn: Zinc, Se: Selenium, Sr: Strontium, Ba: Barium, Pb: Lead. Other minerals that were at background levels and are not shown include cobalt, chromium, manganese, nickel, silver and cadmium. Sodium, calcium and potassium were at high levels for all animals and are not shown.
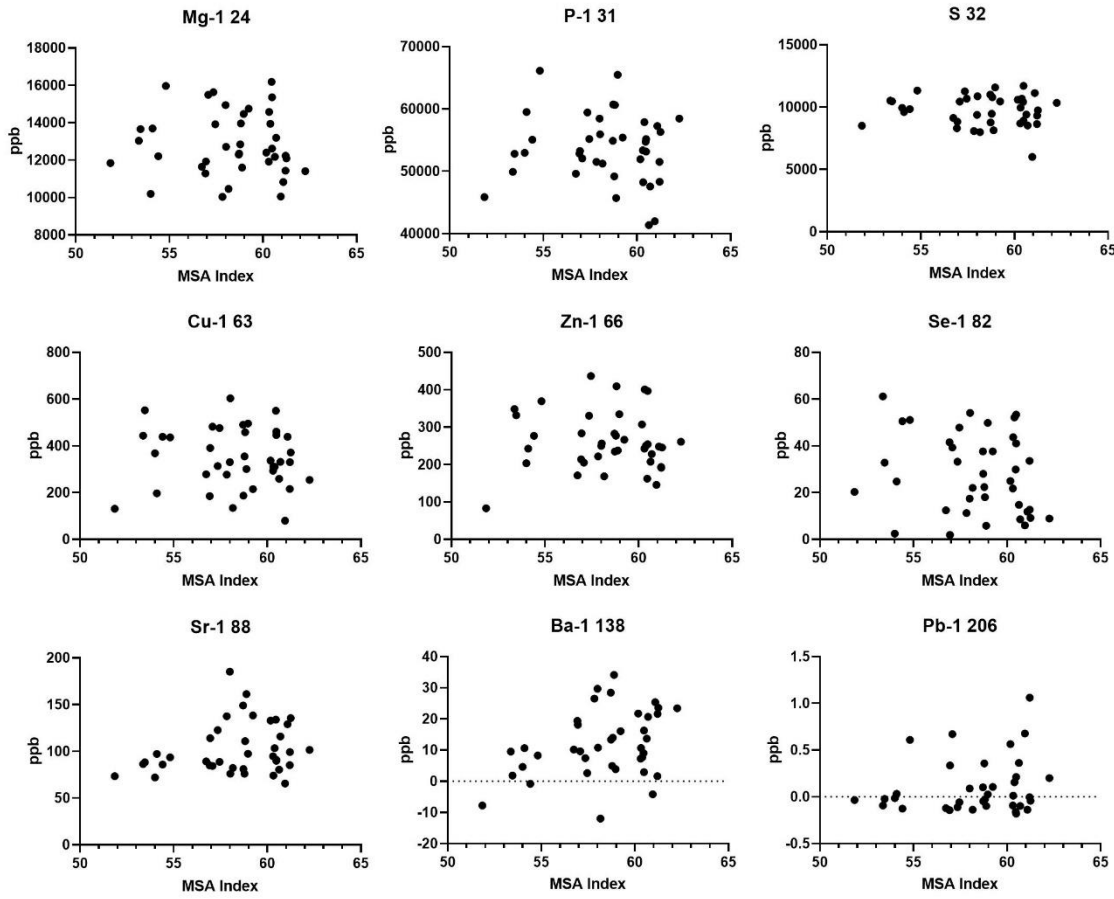
Figure 4.2.21. Scatterplot of trace mineral levels in individual cattle from the feedlot trial versus MSA Index. There were no significant correlations found (Spearman's correlation). Mg: Magnesium, P: Phosphorus, S: Sulfur, Cu: Copper, Zn: Zinc, Se: Selenium, Sr: Strontium, Ba: Barium, Pb: Lead. Other minerals that were at background levels and are not shown include cobalt, chromium, manganese, nickel, silver and cadmium. Sodium, calcium and potassium were at high levels for all animals and are not shown.

4.3 Genetic correlates

4.3.1 Transcriptomic analysis of Feedlot finished cattle

Cattle introduced to feedlots vary in breed, sex, genetic lines, farm of origin, initial body weight, body condition score and previous ration or pasture diet. Given the variation in background conditions it was expected that there would also be significant variation in gene expression. Previous studies have investigated differential gene expression associated with feed efficiency within the liver, muscle, adipose tissue and ruminal mucosa of ruminants (Chen et al., 2011; Connor et al., 2010; Kern et al., 2016; Lindholm-Perry et al., 2020). We sought to identify genes whose expression may be predictive of production outcome either at arrival at feedlot or consistently across the

duration of the finishing process. This study concentrated on feedlot duration within the range of 60-80 days as this duration is commonly used within Australia (Behrendt & Weeks, 2019). The results from this study were incorporated to the meta-analysis in section 4.3.2 to achieve Objective 1b and Objective 2.

A key performance measure of feedlot cattle is average daily gain (ADG). Within feedlots there is significant variation in ADG however within Queensland in Australia, the average steer gains 1.5kg/head/day in comparison to cattle on a pasture based feeding system e.g. Leucaena-grass fed cattle gain approximately 0.7 kg/head/day over a year, whereas grass-only fed cattle gain 0.58 kg/head/day over a year (Emery & Sneath, 2015). This has great economic significance as an animal which can be fed the same quantity but shows increased feed conversion is of more value both in terms of economic return and welfare. Environmentally, it has been established that high ADG results in shorter feedlot stays, with lower CO_2 emissions per kg of weight gain (Yates, 2009). Economically, purchasing of feed is the 2nd largest production costs associated with feedlot systems, accounting for 20% of total costs (Behrendt & Weeks, 2019). From 2015 – 2017 cost of feed was the 2nd largest contributor driving production costs up by US$65 per 100kg (Behrendt & Weeks, 2019). This has a significant impact on productivity of the industry. Consequently, the beef industry is continually researching tools to enhance ADG (Strydom, 2016), however there is a paucity in the literature investigating transcriptome influences on weight gain, immune fitness, animal health and productivity in cattle (Kern et al., 2016).

The Meat Standards Australia (MSA) index score (MLA) was developed by the Australian red meat industry to improve the eating quality consistency of beef and predicts the average eating quality of a beef carcase (range 0-80), where a carcase with a higher MSA index is indicative of higher beef eating quality. The current average overall MSA Index score is 58.59 and scores ≥ 60.28 are the top 25% of eating quality results while ≤55.29 are indicative of the bottom 25% of eating quality. In 2019 and 2020, the average achieved MSA Index score for grain fed cattle was 57.68, an increase of 0.82 from 2018-19 (Packer, 2020). The ideal production outcome would be a selection tool/ score card for cattle with consistent high average daily gain resulting in good to high meat-eating quality. In this study we have selected the MSA index score as a variable associated with a baseline for productivity in terms of the red meat industry (Objective 3)

4.3.1.1   Quality of data

Principle Component Analysis (Fig. 4.3.1 and 4.3.2) illustrates stratification of each array sample according to variance and clearly shows distinct separation between the high and low ADF (Figure 4.3.1) and MSA Index (Figure 4.3.2). The clear distinction of farm associated clusters linked with ADG regardless of breed type indicates a farm of origin effect.
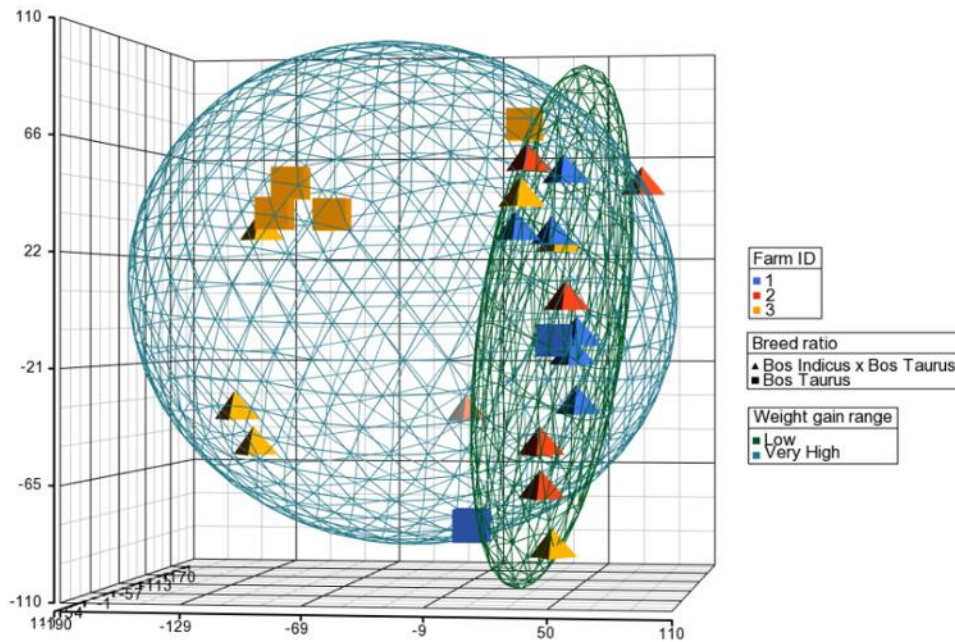
Figure. 4.3.1. Principle Component Analysis of cattle with differing productive performance during finishing at feedlot. The ellipsoid clusters the high performing and the low performing animals (ADG). The shapes represent the breed identities of the cattle. The colours represent the different farms of origin of the animals included in the study.
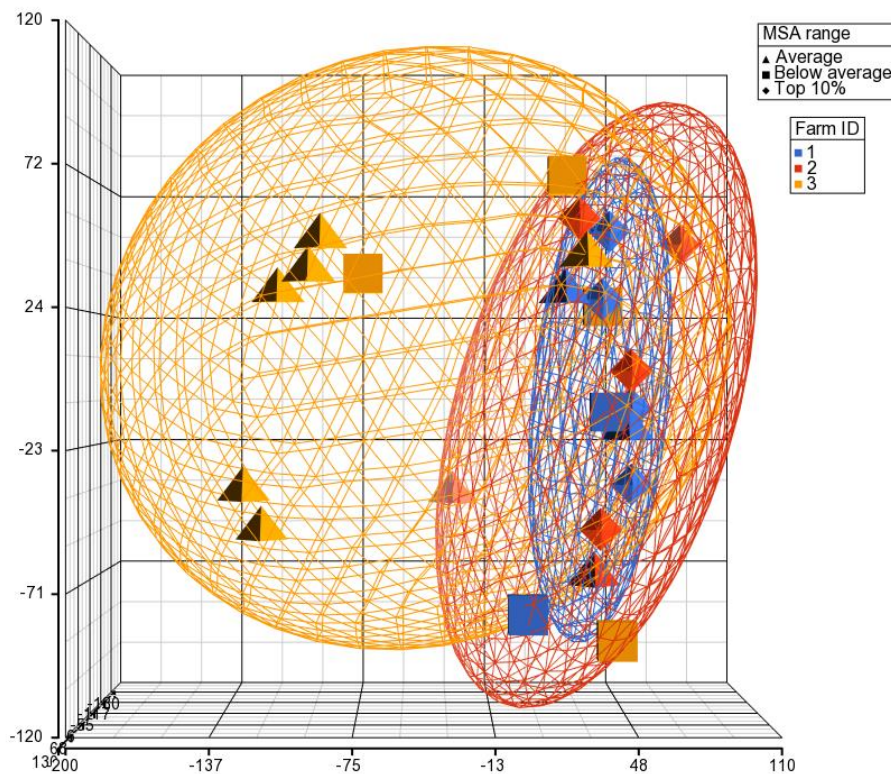
Figure. 4.3.2. Principle Component Analysis of cattle with differing productive performance as measured by MSA index score (shape) and farm of origin (colour and ellipsoid). The cattle with above average MSA index score (≥ 61) are shaped as diamonds, average (57 – 60) are shaped as pyramids and the below average as cubes. The colours represent the different farms of origin of the animals included in the study.

4.3.1.2   Identification of genes associated with ADG

Following statistical analysis of bovine GeneChip™ Gene ST 1.1 Arrays, genes consistently identified as changed across both the sampling time points were associated as having significant differential expression in high percentage ADG opposed to low ADG. Criteria for significance included a p value of ≤0.05 and fold change ≥ 1.4 and 358 genes of interest were identified as having differential gene expression in high ADG cattle opposed to low ADG (**Appendix 8.3**). Of these, the top 15 genes most significantly upregulated, and 15 genes most significantly downregulated in high ADG opposed to low ADG are listed in Table 4.3.1, highlighting p-value and fold change (FC) for each gene. Significance of regulation was ranked through FC, where a negative FC represents down regulation and a positive FC value represents upregulation in the high ADG group opposed to low ADG.

Table 4.3.1. Differentially upregulated and downregulated genes expressed in high ADG cattle at both feedlot day 0 and day 76, transcript. The data is presented with p-value and fold change (FC) for each gene and if annotated, RefSeq number and gene symbol. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**.

Transcript ID	Gene Symbol	RefSeq	Day 0		Day 76	
			p-value	FC	p-value	FC
12889437	ADAMDEC1	NM_001206371	1.84E-02	-3.1	1.33E-02	-1.7
12802789	BOLA	XM_002697329	3.25E-02	-4.4	3.25E-02	-4.5
12805748	BOLA	NM_001038518	3.32E-02	6.3	2.91E-02	6.6
12687880	BRB	NM_173891	5.15E-02	-2.5	2.83E-02	-2.9
12805720	C2	NM_001034492	4.43E-03	2.5	2.98E-03	2.7
12760963	CCL3	NM_174511	1.30E-02	2.7	3.81E-02	2.2
12862491	CLEC4A	NM_001191510	1.61E-02	-2.2	1.60E-02	-2.2
12862473	CLEC4D	NM_001193117	6.62E-03	-1.9	1.73E-02	-1.7
12865991	CLEC4E	XM_002687823	1.19E-02	-2.6	3.62E-03	-3.1
12842594	**CSF1**	**NM_174026**	**8.46E-04**	**3.2**	**5.96E-03**	**2.5**
12717119	CST7	XM_002692174	3.46E-03	2.8	9.05E-03	2.4
12845442	FCRL6	XM_002685897	3.95E-03	3.3	1.13E-02	2.8
12835548	GBP2	XM_010803354	3.09E-02	-2.9	3.09E-02	-3.8
12704196	GNLY	AY245798	5.26E-02	4.2	5.47E-02	4.2
12792134	**GZMB**	**XM_002696646**	**1.10E-03**	**4.8**	**4.11E-02**	**2.5**
12823962	IDO2	XM_010820353	3.86E-03	2.7	4.82E-03	2.6
12738469	**IL10**	**NM_174088**	**1.34E-02**	**3.2**	**3.68E-02**	**2.6**
12857041	KLRA1	NM_174376	1.32E-02	2.8	5.92E-03	1.9
12856272	**KLRC1**	**NM_001168587**	**1.63E-02**	**-2.8**	**1.04E-02**	**-3.1**
12703589	LOC100300483	XM_002691364	3.82E-03	3.3	1.93E-02	2.5
12708928	LOC101902625	XM_005213909	5.17E-02	-2.2	5.17E-02	-2.4
12906936	LOC101904998	XM_010800823	7.36E-03	3.7	4.55E-02	2.5
12699011	LOC505058	XR_809793	1.26E-02	-2.3	3.64E-03	-2.7
12765836	LOC781298	XM_001249617	4.06E-02	-1.6	3.82E-02	-1.6
12692359	MGC148318	NR_038117	3.84E-02	-1.7	4.06E-02	-1.7
12748219	OAS1Y	NM_001040606	3.26E-02	2.8	7.88E-03	2.3
12855928	RGS2	NM_001075596	1.78E-03	-2.9	4.22E-02	-1.9
12704418	RSAD2	NM_001045941	2.21E-02	2.7	1.06E-02	1.8
12708832	STK24	NM_001099950	1.27E-02	-1.7	7.91E-03	-1.7
12908904	ZNF711	NM_001205925	9.72E-04	-1.7	4.05E-03	-1.6

Hierarchical clustering illustrates the clear variation in gene expression between proposed immune fitness markers for high ADG achieving cattle in comparison to the low ADG cohort at induction (Fig. 4.3.3).

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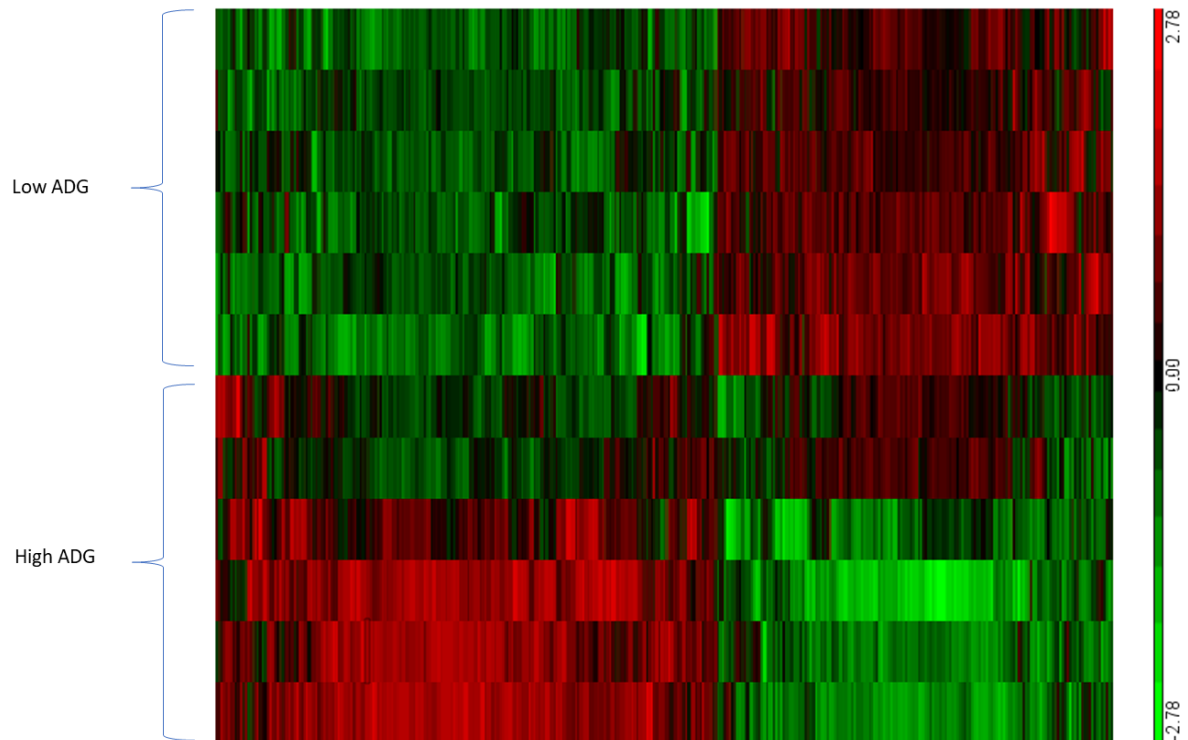
Figure 4.3.3. Hierarchical Clustering of genes expressed in the 12 high and low ADG individuals, displayed as a heat map. Each row represents an individual animal and each column an individual gene. Green represents a negative fold change (downregulated), black represents no fold change, and red a positive fold change (upregulated).

4.3.1.3 Identification of genes associated with MSA index score – a baseline for red meat quality.

Following statistical analysis of the GeneChip™ derived data, 226 genes meeting the criteria for differential expression (FDR ≤0.05 and a FC ≥1.5), at induction (day 0) or consistently across both sampling times (Day 0 and day 76) are associated to cattle that achieved a high MSA index score in comparison to those cattle who achieved low score are listed in **Appendix 8.3**. Of these, the top 15 genes most significantly upregulated, and 15 genes most significantly downregulated in cattle achieving the top 25% MSA index score at day 0 and day 76 opposed to low MSA index score are listed in Table 4.3.2, highlighting p-value and fold change (FC) for each gene. Significance of regulation was ranked through FC, where a negative FC represents down regulation and a positive FC value represents upregulation in the high ADG group opposed to low ADG.

These genes represent a baseline measure for optimal immune fitness linked to red meat quality.

Table 4.3.2. Differentially upregulated and downregulated genes expressed in cattle achieving the top 25% MSA index score at day 0 and day 76. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**.

Transcript ID	Gene Symbol	RefSeq	Day 0		Day 76	
			p-value*	FC*	p-value	FC
12801188	BOLA-DQB	NM_001012676	3.61E-06	-9.6	1.48E-05	-7.1
12849084	PRSS2	XM_010804532	4.27E-02	-7.9	5.87E-02	-6.7
12713539	BPI	NM_173895	9.79E-03	-7.5	3.94E-01	-1.8
12811041	LOC100297063	XR_815336	2.06E-02	-3.7	3.48E-01	-1.6
12804634	BOLA-DQA5	NM_001012675	5.12E-05	-3.2	1.68E-03	-2.2
12675243		---	3.16E-02	-3	2.44E-02	-3.2
12742542	LOC511354	AF023842	3.11E-02	-2.9	9.62E-02	-2.2
12856272	**KLRC1**	**NM_001168587**	**5.67E-01**	**-2.9**	**7.11E-01**	**-1.4**
12678021		---	6.23E-04	-2.8	7.92E-02	-1.5
12738469	**IL10**	**NM_174088**	**1.74E-01**	**-2.7**	**3.53E-01**	**-2**
12850545	TARP	XM_003585963	2.48E-02	-2.7	2.44E-01	-1.6
12915337		---	2.63E-02	-2.7	2.07E-01	-1.7
12688730	LOC509034	XM_002691069	4.64E-02	-2.7	3.42E-01	-1.6
12802801	BoLA	NM_001114855	4.65E-03	-2.7	1.62E-03	-3.1
12803584	LOC528329	XM_010818460	3.11E-02	-2.6	3.79E-01	-1.4
12792134	**GZMB**	**XM_002696646**	**2.27E-02**	**-2.2**	**6.72E-02**	**-1.4**
12812811	**HSPB1**	**NM_001025569**	**6.51E-02**	**-1.9**		
12842594	**CSF1**	**NM_174026**	**2.00E-02**	**-1.5**	**3.00E-02**	**-1.5**
12870628	PF4	NM_001101062	4.21E-02	3.3	2.40E-01	1.9
12914029		---	1.12E-02	3.6	1.27E-01	2
12687084	PIGP	NM_001077022	2.34E-02	3.6	7.97E-02	2.6
12792366	LOC512440	NM_001101956	3.15E-02	4.4	2.94E-03	9.7
12677569		---	2.67E-02	4.5	4.39E-01	1.6
12676565		---	1.44E-02	5.1	5.22E-02	3.4
12704196	GNLY	AY245798	3.61E-06	5.2	4.87E-03	17.7
12678067		---	1.57E-02	5.5	4.66E-02	3.8
12911293		---	4.27E-02	6	5.54E-01	4
12755563	LOC100337097	XM_010800761	9.79E-03	6.5	8.18E-02	6.9
12903084		---	2.06E-02	12.6	3.73E-01	13.4

*The data is presented with p-value and fold change (FC) for each gene as well as transcript ID and if the annotation is known, RefSeq number and gene symbol.

4.3.1.4 Identification of genes common to cattle with high ADG and top MSA index score

Following statistical analysis of the GeneChip™ derived data, 54 genes meet the criteria for differential expression (FDR ≤0.05 and a FC ≥1.5) for cattle achieving both high ADG and a high MSA

index score Table 4.3.3. The annotation for many of these predicted genes are unknown however these genes are suggested as candidates of baseline of optimum red meat quality in cattle finished in the feedlot (Objective 3).

Table 4.3.3. Differentially upregulated and downregulated genes expressed in cattle achieving the top 25% MSA index score and high ADG. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**.

Transcript ID	Gene Symbol	RefSeq	Consistent	
			p-value*	FC*
12713539	BPI	NM_173895	0.02767	-10.9986
12814535	TMEM159	NM_001080361	0.00071	-6.06335
12915261		---	0.0099	-5.33439
12675243		---	0.0456	-4.34653
12678021		---	0.0027	-3.39797
12750220	LOC506868	XM_002683772	0.01297	-3.03392
12726957	APOC3	ENSBTAT00000016453	0.00696	-2.95534
12856272	**KLRC1**	**NM_001168587**	**0.00125**	**-2.90301**
12852821	ISPD	XM_002686679	0.00125	-2.83122
12768492		---	0.01439	-2.78927
12899089	SH3BGRL2	NM_001083791	0.00617	-2.56094
12695043	AQP9	NM_001205833	0.01712	-2.4593
12859585	CLEC6A	NM_001034479	0.01893	-2.28013
12834341	SLC29A2	NM_001103269	0.01199	-2.07871
12801188	BOLA-DQB	NM_001012676	0.2667	-1.61128
12804195	SMIM13	NM_001135578	0.0231	1.53525
12766826	MIR451	NR_031059	0.02968	1.56154
12852248	ARL4A	NM_001076985	0.03437	1.70607
12915191		---	0.04266	1.72646
12802588	CAGE1	XM_002697621	0.04628	1.73079
12885817	HBEGF	NM_001144090	0.01976	1.77539
12817362	CYP3A5	NM_001075888	0.00106	1.81582
12698253		---	0.00224	1.87952
12913769		---	0.00777	1.88694
12870858	CXCL13	NM_001015576	0.00835	1.93871
12797198	ARPP21	NM_001076950	0.02587	1.97345
12754721		---	0.02577	2.07116
12875557	GPR125	XM_002688170	0.02475	2.13294
12697609	GCNT4	XM_005200869	0.04804	2.17065
12913695		---	0.00082	2.41156
12677429		---	0.02952	2.4476
12801474	BOLA	DQ140374	0.0794	2.5558
12887146	F2RL3	NM_001076138	0.00675	2.59423
12683738	SOX14	NM_001163781	0.01719	2.62044

Transcript ID	Gene Symbol	RefSeq	Consistent	
			p-value*	FC*
12676507		---	0.04005	2.64039
12677765		---	0.04606	2.77796
12910525	LOC104968579	XM_001250462	0.01403	2.82259
12843576	FCER1A	NM_001100310	0.00125	2.98909
12713427	LOC100335935	XM_010811259	0.02848	3.03852
12912807		---	0.00999	3.08527
12760468	MIR2335	NR_030856	0.03752	3.14249
12677317		---	0.01803	3.42513
12836228	S100A13	NM_205800	0.0055	3.60131
12793338	IFI27L2	XM_010817266	0.0112	3.61446
12693777	BMP4	NM_001045877	0.00158	4.0592
12677121		---	0.00628	4.32878
12739271		---	0.01139	4.35913
12912345		---	0.02111	4.55465
12704196	GNLY	AY245798	0.22226	4.78134
12678069		---	0.03287	4.91839
12913101		---	0.0003	5.58044
12677929		---	0.00669	5.97033
12676565		---	0.04542	6.41128
12678067		---	0.032	8.42051

* The data is presented with p-value and fold change (FC) for each gene as well as transcript ID and where annotation is known, RefSeq number and gene symbol.

4.3.1.5   Pathway analysis of genes of interest (cattle)

Not all identified genes were mapped to a known functional or biological pathway. Of the 444 successfully known mapped genes, Pathway analysis seeks to identify genes to groups and then finding overrepresented groups in filtered gene lists using Fisher's test. Significant pathways are those with an enrichment score ≥ 3 or p-value ≤0.05 and 18 significant key canonical pathways were associated with the differential expressed genes in cattle that achieved high ADG during the feedlot finishing process and at slaughter their carcase was classed withing the top 25% MSA index scoring. The analysis was weighted to genes differentially expressed at induction (day 0) and these are primarily related to metabolic and immune function (Table 4.3.4).

Table 4.3.4: Canonical pathways identified in high ADG & top 25% MSA scoring cattle generated using Partek Pathway function within Partek Genomics Suite (Partek Inc).

Pathway Name	Enrichment Score	Enrichment p-value	# genes regulated
Metabolic pathways	3.62	2.20E-02	31
Cell adhesion molecules (CAMs)	7.78	4.20E-04	11
Endocytosis	3.61	7.38E-02	9
Antigen processing and presentation	8.50	2.03E-04	8
Cytokine-cytokine receptor interaction	3.42	3.24E-02	8
Hematopoietic cell lineage	5.08	6.21E-03	7
Th17 cell differentiation	4.68	9.32E-03	7
Phagosome	3.27	5.13E-02	7
Intestinal immune network for IgA production	7.51	5.49E-04	6
Th1 and Th2 cell differentiation	4.13	1.61E-02	6
Cell cycle	3.11	4.44E-02	6
Steroid biosynthesis	7.28	6.86E-04	4
Mannose type O-glycan biosynthesis	6.88	1.02E-03	4
Terpenoid backbone biosynthesis	4.69	9.15E-03	3
Vitamin digestion and absorption	4.18	1.52E-02	3
Cholesterol metabolism	3.57	2.65E-02	3
Arginine biosynthesis	4.74	6.48E-02	2
Other types of O-glycan biosynthesis	3.56	7.74E-02	2

Focus genes attributed to antigen presentation pathways were overrepresented in high production measure cattle with a predicted increase in activation of MHC class II CD4^{+} T lymphocyte antigen presentation (Fig. 4.3.4). Fourteen focus genes aligned to carbohydrate metabolism, cellular development and protein synthesis were overrepresented in high ADG cattle with predicted overall increase in activation (Fig. 4.3.5) whereas 13 focus genes aligned to lipid metabolism, small molecular biochemistry, vitamin and mineral metabolism were overrepresented in high ADG cattle with a predicted overall decrease in activation (Fig. 4.3.6).
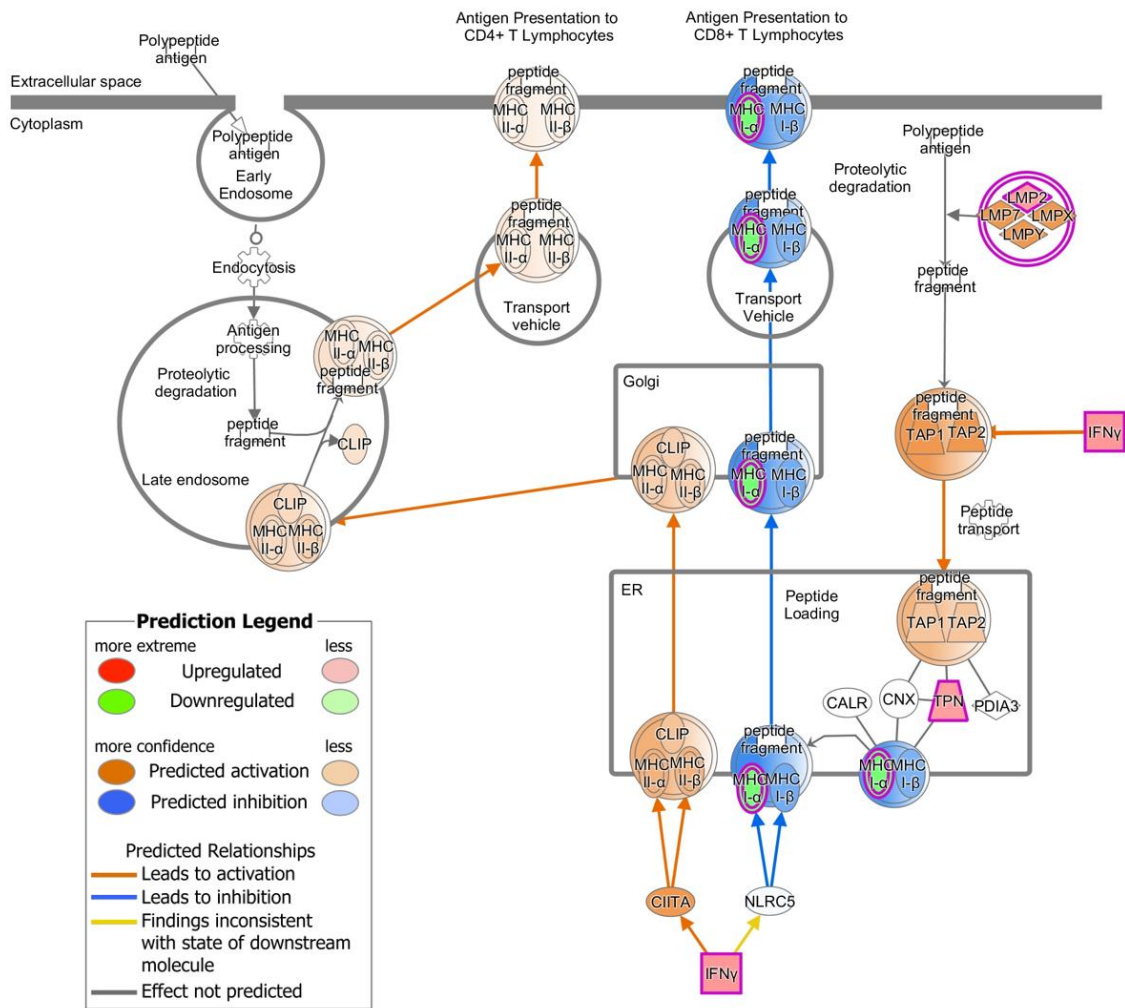
Figure 4.3.4: Activation of MHC class II Antigen presenting pathway and inhibition of MHC class I pathway in high ADG cattle. Figure displaying the up-regulation of IFN-gamma with an overlay of the predicted activation of MHCII pathways and inhibition of MHCI pathways. Red = up-regulated, green = downregulated, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.
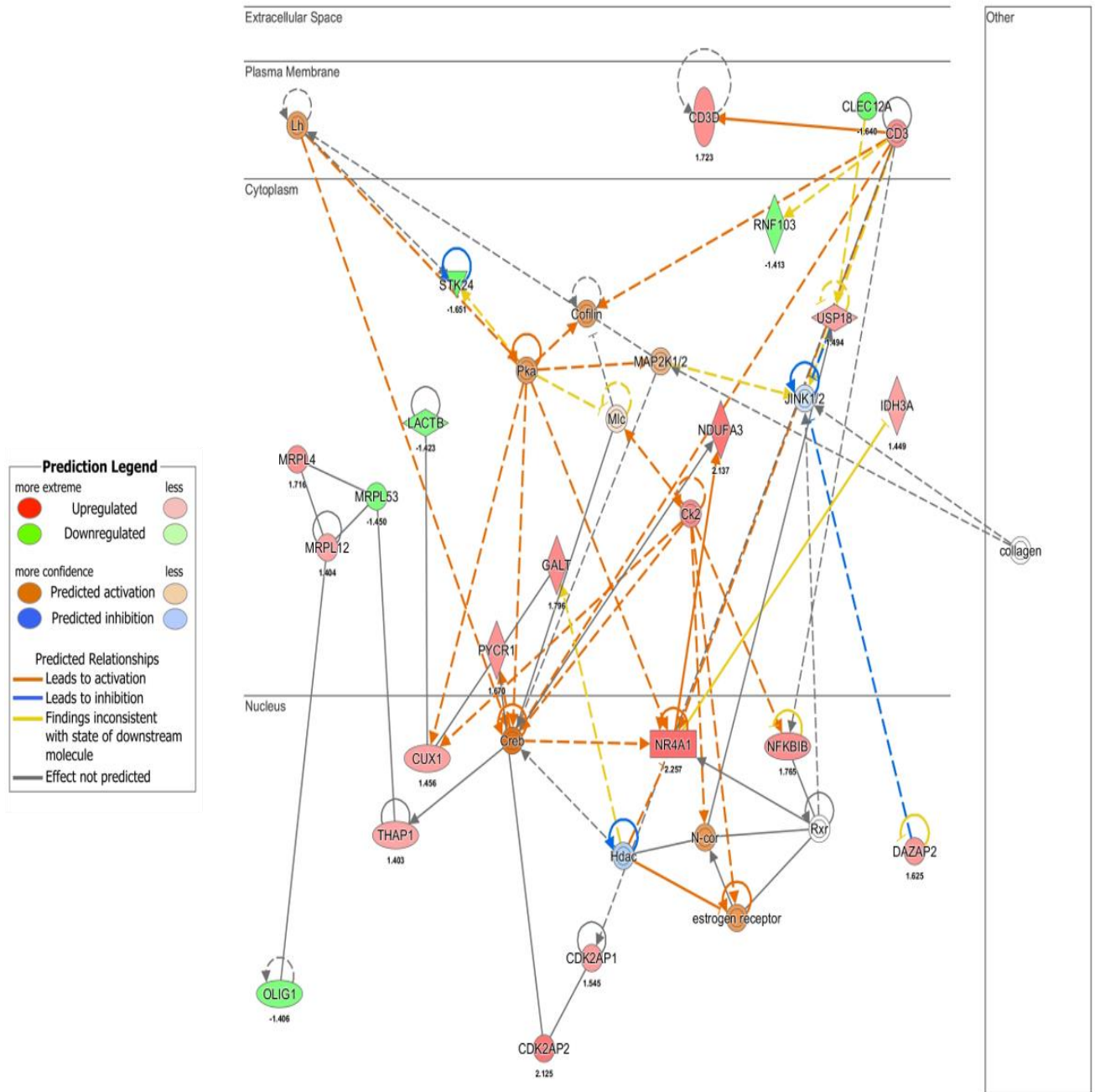
Figure 4.3.5. Activation of regulatory network controlling Carbohydrate Metabolism, Cellular development, and protein synthesis in high ADG achieving cattle. 21 focus molecules were identified to be overrepresented within this pathway. This figure displays the regulatory network controlling oxidative phosphorylation, ATP synthesis, ribosomal protein production and others. Overlaid is the predicted activation or inhibition by overrepresented genes in high ADG cattle. Red = up-regulated, green = downregulated, orange = predicted activation, blue = predicted inhibition, grey = effect not predicted. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.
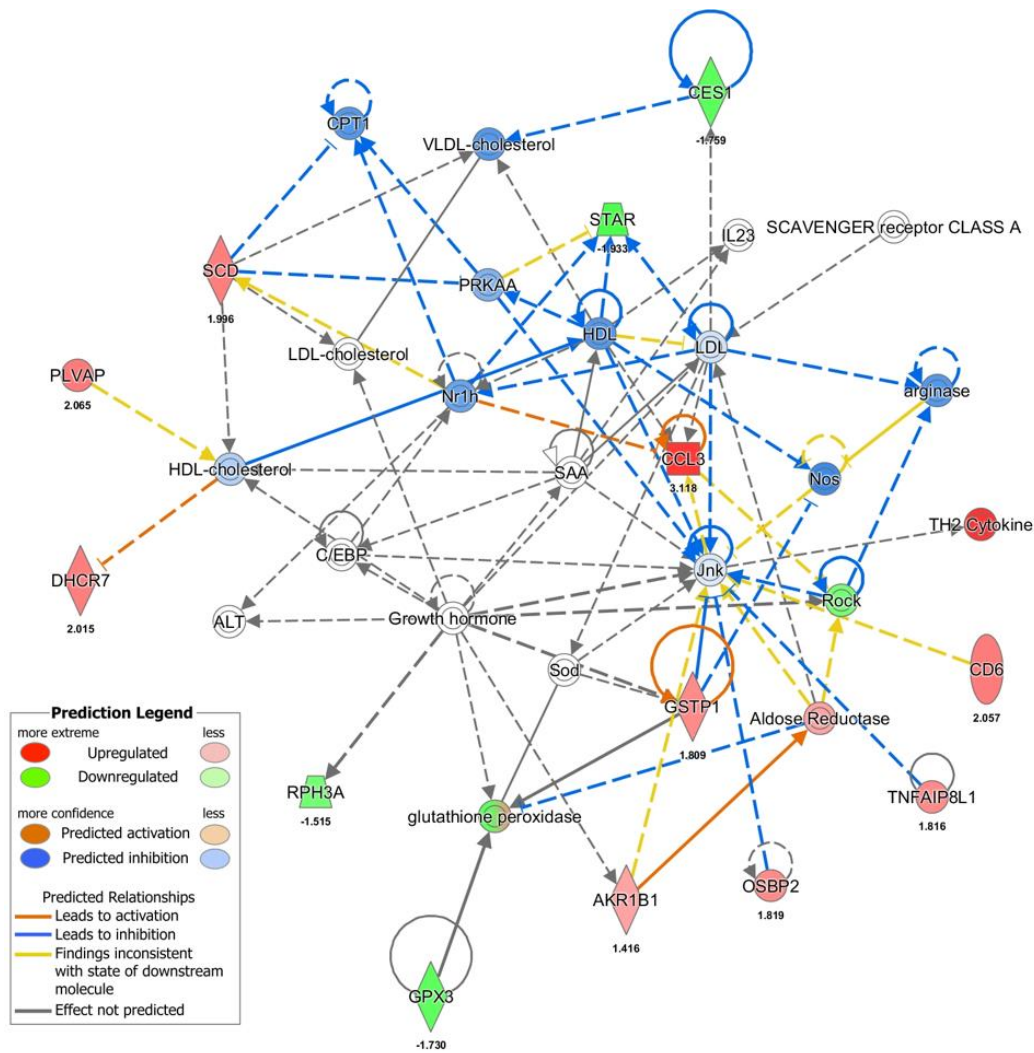
Figure 4.3.6. Differential inhibition of genes controlling Lipid Metabolism, small molecule biosynthesis, vitamin and mineral metabolism was mapped to high production measure achieving beef cattle. 14 focus molecules were identified to be overrepresented within this pathway. This figure displays the regulatory network controlling HDL-cholesterol, VLDL cholesterols, HDL, CCL3, Aldose reductase, glutathione peroxidase. Overlaid is the predicted activation or inhibition by overrepresented genes in high ADG cattle. Red = up-regulated, green = downregulated, orange = predicted activation, blue = predicted inhibition, grey = effect not predicted. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.

Upstream regulator analysis identified Interferon alpha (IFNα), Interleukin 1beta (IL-1β), Interferon gamma (IFNγ), STAT1, IRF7 and IFNA2 as potential candidates responsible for the pathways described in the dataset.

4.3.2   Meta- analysis of transcriptomic datasets

4.3.2.1   Selection of datasets

Seventeen datasets met the selection criteria, which included availability of intensity calculations on the pixel values of the gene expression data files (CEL. files), more than 5 samples, annotation comprising of comparable variables (e.g. timepoint, varying treatments including provision of a control cohort) and exposure to relevant stressors. Suitable datasets (Table 4.3.5) were associated to sheep or cattle exposed to pathogens *Brucella ovis* (sheep), *Mycobacterium bovis* (cattle), *Mycobacterium avium* subsp. *paratuberculosis* (sheep and cattle), and *Rhipicephalus microplus* (sheep and cattle), Pestivirus (cattle), *Neotyphodium coenophialum* (cattle), acidosis, high and low intramuscular fat, varying feed conditions including grass-fed, concentrated feed and feedlot pelleted ration (cattle), environmental stress conditions including cold exposure and physical and psychological stress (cattle).

There were no suitable goat gene expression datasets available.

The raw data files (CEL. format files) from the following studies were downloaded from GEO (Table1): GSE11835 (Smirnova et al., 2009), GSE33359 (Killick et al., 2011), GEO57907 (Carvalho et al., 2014), GSE23894 (Liao, Boling & Matthews, 2015) GSE15342 (Piper et al., 2008; Piper et al., 2009), GSE162156 (Jiminez et al., 2021), GSE146971 (Del Corvo et al., 2020), GSE119912 (unpublished), GSE13725 (Piper et al., 2009) ,GSE79827 (Xu et al., 2017), GSE52145 (Sweeney et al., 2016), GSE75347 (Komolka et al., 2016), GSE75348 and GSE39006 (Albrecht et al., 2016), GSE17849 (Steele et al., 2011), GSE70248 (Bai et al., 2020), GSE94777 (Crocker Cunningham et al., 2018; Cunningham et al., 2018; Lindholm-Perry et al., 2017), GSE35615 (Azevedo et al., 2017). In addition, datafiles generated by our group were added to the larger datafile: GSE114384/P.PSH.0311 (Purdie et al., 2019a) P.PSH.0297 (Purdie et al., 2012), P.PSH.0576 (in preparation for publication) and P.PSH.0816 (see Section 3.1).

Table 4.3.5: Characteristics of selected microarrays for analysis

Series accession #	Reference	Platform ID	Characteristics	
GSE11835	(Smirnova et al., 2009)	GPL2112	Number	18
			Breed	Holstein
			Treatment	*Pestivirus* (96B2222)
			Timepoints	2
			Feed type	Unspecified
			Sample source	WBC
GSE33359	(Killick et al., 2011)	GPL2112	Number	16
			Breed	Holstein Friesian cross
			Treatment	*Mycobacterium bovis*
			Timepoints	1
			Feed type	Grass
			Sample source	Whole blood
GSE57907	(Carvalho et al., 2014)	GPL2112	Number	13
			Breed	Gyr x Holstein
			Treatment	*Rhipicephalus microplus*
			Timepoints	3
			Feed type	Unspecified
			Sample source	Skin
GSE15342	(Piper et al., 2009)	GPL2112	Number	12
			Breed	Brahman, Holstein-Friesian
			Treatment	*Rhipicephalus microplus*
			Timepoints	1
			Feed type	Unspecified
			Sample source	Skin
GSE162156	(Jiminez et al., 2021)	GPL23295	Number	43
			Breed	Mixed beef cattle
			Treatment	BRD
			Timepoints	1
			Feed type	Unspecified
			Sample source	WBC
GSE119912	Unpublished	GPL25574	Number	32
			Breed	Normand
			Treatment	Physical and psychological stress (social isolation, transport, deprivation of food and water)
			Timepoints	1
			Feed type	Unspecified
			Sample source	Muscle (longissimus thoracis and semitendinosus)
GSE13725	(Piper et al., 2009)		Number	6
			Breed	Brahman, Holstein-Friesian
			Treatment	*Rhipicephalus microplus*

Series accession #	Reference	Platform ID	Characteristics	
			Timepoints	1
			Feed type	
			Sample source	WBC
GSE146971	(Del Corvo et al., 2020)	GPL19172	Number	13
			Breed	Red Piedmont
			Treatment	Cortisol expression (high, low)
			Timepoints	Unspecified
			Feed type	Grass
			Sample source	Milk
GSE52145	(Sweeney et al., 2016)	GPL2112	Number	14
			Breed	Charolais-Limousin
			Treatment	Feed variation
			Timepoints	1
			Feed type	Pasture or pellet/Grain for 12 months
			Sample source	Muscle (longissimus dorsi)
GSE23894	(Liao, Boling & Matthews, 2015)	GPL2112	Number	19
			Breed	Angus
			Treatment	*Neotyphodium coenophialum*
			Timepoints	1
			Feed type	grass
			Sample source	Liver
GSE75347	(Komolka et al., 2016)	GPL2112	Number	20
			Breed	Holstein x Charolais
			Treatment	Feed variation (high, low muscle variation)
			Timepoints	1
			Feed type	silage-based diet
			Sample source	Muscle (longissimus dorsi)
GSE75348	(Albrecht et al., 2016)	GPL2112	Number	6
			Breed	Japanese black, Holstein Freisian
			Treatment	Feed variation
			Timepoints	1
			Feed type	High energy diet
			Sample source	Muscle (longissimus dorsi)
GSE17849			Number	6
			Breed	Holstein
			Treatment/ observation	Feed variation induced acidosis
			Timepoints	3
			Feed type	Grass, Grain
			Sample source	Rumen papillae
GSE39006	(Albrecht et al., 2016)	GPL2112	Number	6

Series accession #	Reference	Platform ID	Characteristics	
			Breed	Japanese black, Holstein
			Treatment	Feed variation
			Timepoints	1
			Feed type	High energy diet
			Sample source	Subcutaneous, intramuscular fat
GSE79827	(Xu et al., 2017)	GPL2112	Number	30
			Breed	Sanhe
			Treatment	Environmental stress (cold exposure)
			Timepoints	Pre and post exposure to cold
			Feed type	Mixed ration
			Sample source	WBC
GSE70248	(Bai et al., 2020)	GPL15749	Number	16
			Breed	Angus
			Treatment /variables	Feed Variation
			Timepoints	1
			Feed type	Pasture, Grain
			Sample source	Muscle (longissimus dorsi)
GSE94777	(Crocker Cunningham et al., 2018)	GPL20768	Number	12
			Breed	Angus Hereford X
			Treatment /variables	Feed variation (nutrient restricted vs ad libitum)
			Timepoints	2
			Feed type	high, low grain
			Sample source	Adipose tissue
GSE35615	(Carvalho et al., 2014)	GPL2112	Number	12
			Breed	Santa Ines
			Treatment	*Brucella ovis*
			Timepoints	3
			Feed type	Unspecified
			Sample source	WBC
P.PSH0816		GPL20768	Number	12
			Breed	Mixed beef cattle
			Treatment	Environmental stress (feedlot)
			Timepoints	2
			Feed type	Grain
			Sample source	WBC
P.PSH297	(Purdie et al., 2012)	GPL2112	Number	20
			Breed	Holstein
			Treatment	MAP
			Timepoints	5
			Feed type	Grass

Series accession #	Reference	Platform ID	Characteristics	
			Sample source	WBC
GSE114384 (P.PSH.0311)	(Purdie et al., 2019a)	GPL2112	Number	25
			Breed	Merino
			Treatment	MAP
			Timepoints	5
			Feed type	Grass
			Sample source	WBC
P.PSH.0576.1		GPL2112	Number	25
			Breed	Merino
			Treatment	MAP (Gudair vaccinated)
			Timepoints	2
			Feed type	Grass
			Sample source	WBC

4.3.2.2   Selection of gene expression correlates of immune fitness (Cattle)

Following statistical analysis of the GeneChip™ derived data, 165 genes met the criteria for differential expression (FDR ≤0.05 and a FC ≥1.5), with disease resilience in cattle across multiple breeds, sample sources and disease exposure states and 355 genes were associated with optimal production (high ADG) in intensive grain fed/mixed feed (**Appendix 8.4**). Of these, the top 15 genes most significantly upregulated, and 15 genes most significantly downregulated in cattle achieving resilience to disease are listed in Table 4.3.6, and those associated with optimal production (high ADG) in intensive grain fed/mixed feed are listed in Table 4.3.7. The lists highlight p-value and fold change (FC) for each gene. Significance of regulation was ranked through FC, where a negative FC represents down regulation and a positive FC value represents upregulation in the high ADG group opposed to low ADG. Genes whose expression has been validated by qPCR are indicated in **bold**. Genes previously validated as markers of disease exposure in MAP exposed cattle (P.PSH.0276 final report) are *italicised*.

Thirty-five genes were identified with common expression between disease/stress resilient cattle and those capable of achieving a high weight gain in the production system (Table 4.3.8). These genes are ideal candidates for correlates of fitness (Objective 1b)

Table 4.3.6. Differentially upregulated and downregulated genes expressed in cattle achieving resilience to disease and environmental stress. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**. Genes previously validated as markers of disease exposure in MAP exposed cattle (P.PSH.0276 final report) are *italicised*.

Gene Symbol	Ensembl	p-value*	Fold-Change*
BOLA-DQA2	---	*1.67E-05*	*-6.2*
BOLA-DQA5	---	*2.00E-07*	*-5.9*
DNER	---	5.72E-15	-5.3
BOLA-DQA1	---	*5.99E-06*	*-5*
BLA-DQB	*ENSBTAG00000019588*	*2.27E-04*	*-4.3*
BOLA-DQB	*ENSBTAG00000021077*	*6.32E-08*	*-4.2*
GSTO1	ENSBTAG00000003989	4.40E-10	-2.5
LOC100851058	---	4.84E-03	-2.5
IMPA2	**ENSBTAG00000043951**	**1.68E-05**	**-2.4**
CSF1	**ENSBTAG00000000283**	**3.95E-06**	**-2.3**
HSPB1	**ENSBTAG00000011969**	**6.02E-03**	**-2.3**
LOC509034	---	3.58E-10	-2.3
PTGR1	**ENSBTAG00000011027**	**1.99E-05**	**-2.2**
CYP2B6	ENSBTAG00000003871	6.57E-04	-1.9
LOC510382	---	1.15E-06	-1.9
LOC101906310	---	6.21E-05	1.7
TYRP1	ENSBTAG00000020985	7.21E-03	1.7
FOS	ENSBTAG00000004322	2.76E-03	1.8
LOC786987	---	2.32E-04	1.8
MIF	ENSBTAG00000007375	2.62E-05	1.8
BOLA-N	*ENSBTAG00000020116*	*2.90E-02*	*1.9*
JSP.1	---	1.18E-05	1.9
SYT4	ENSBTAG00000001801	3.09E-06	2
IGK	---	4.19E-08	2.2
LOC100337108	---	1.99E-04	2.3
PRSS2	ENSBTAG00000021565	3.24E-04	2.4
IGLL1	ENSBTAG00000031160	3.47E-05	2.6
IGHG1	---	3.66E-05	2.9
BOLA	---	*5.55E-04*	*3.2*
KLRC1	**ENSBTAG00000054633**	**2.27E-07**	**3.4**

* The data is presented with p-value and fold change (FC) for each gene as well Ensembl number and gene symbol.

Table 4.3.7. Differentially upregulated and downregulated genes expressed in cattle capable of achieving a high weight gain in the production system. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**. Genes previously validated as markers of disease exposure in MAP exposed cattle (P.PSH.0276 final report) are *italicised*.

Transcript ID	Gene Symbol	RefSeq	p-value*	Fold change*
12802789	*BOLA*	*XM_002697329*	*3.3E-02*	*-4.5*
12835548	GBP2	XM_010803354	3.1E-02	-3.4
12856272	**KLRC1**	**NM_001168587**	**1.3E-02**	**-3.0**
12865991	CLEC4E	XM_002687823	7.8E-03	-2.9
12687880	BRB	NM_173891	4.0E-02	-2.7
12699011	LOC505058	XR_809793	8.3E-03	-2.5
12889437	ADAMDEC1	NM_001206371	1.6E-02	-2.4
12855928	RGS2	NM_001075596	2.2E-02	-2.4
12708928	LOC101902625	XM_005213909	5.2E-02	-2.3
12862491	CLEC4A	NM_001191510	1.6E-02	-2.2
12875788	IGFBP7	NM_001102300	1.1E-02	-2.0
12735655	LOC515150	XM_002693873	2.4E-02	-1.9
12862473	CLEC4D	NM_001193117	1.2E-02	-1.8
12805255	*BOLA-NC1*	*NM_001105616*	*1.4E-02*	*-1.7*
12692359	MGC148318	NR_038117	4.0E-02	-1.7
12754367	MRPS12	XM_005219040	1.2E-02	2.4
12747162	OAS2	NM_001024557	3.8E-02	2.4
12760963	CCL3	NM_174511	2.6E-02	2.5
12748219	OAS1Y	NM_001040606	2.0E-02	2.6
12717119	CST7	XM_002692174	6.3E-03	2.6
12805720	C2	NM_001034492	3.7E-03	2.6
12793812	LOC617313	XM_002696688	2.6E-03	2.6
12823962	IDO2	XM_010820353	4.4E-03	2.7
12842594	**CSF1**	**NM_174026**	**3.4E-03**	**2.9**
12703589	LOC100300483	XM_002691364	1.1E-02	2.9
12738469	**IL10**	**NM_174088**	**2.5E-02**	**2.9**
12845442	FCRL6	XM_002685897	7.5E-03	3.1
12906936	LOC101904998	XM_010800823	2.7E-02	3.1
12704196	GNLY	AY245798	5.4E-02	4.2
12788092	**GZMB**	**NM_001099095**	**0.0335**	**4.8**

* The data is presented with p-value and fold change (FC) for each gene as well as transcript ID, Refseq number and gene symbol.

Table 4.3.8. Differentially upregulated and downregulated genes with common expression between disease/stress resilient cattle and those capable of achieving a high weight gain in the production system. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**. Genes previously validated as markers of disease exposure in MAP exposed cattle (P.PSH.0276 final report) are *italicised*.

Gene Symbol	Disease resilience		High weight gain	
	p-value	**Fold Change**	**p-value***	**Fold Change***
ASPH	5.70E-09	-1.7	3.30E-03	-2.4
BACE2	2.50E-07	-1.4	1.30E-01	-2.2
CD1B	3.30E-08	1.7	2.10E-02	2
CD38	1.40E-07	1.4	3.10E-01	2
DHX58	8.60E-06	1.4	2.90E-02	2.8
DMBT1	4.80E-06	-1.4	3.10E-01	-2.1
EIF4EBP1	2.70E-11	1.4	9.80E-02	2.1
FILIP1	5.70E-09	-1.4	5.10E-02	-3.5
GZMB	**4.70E-04**	**1.5**	**1.10E-03**	**4.8**
BOLA-DQA1	*1.10E-08*	*-4.7*	*5.80E-01*	*-2.6*
ICAM2	2.00E-06	1.3	3.50E-03	2.2
IFI44	1.10E-04	1.6	2.40E-01	3
IGF2BP3	1.80E-08	2	6.70E-02	2.3
IGFBP2	4.80E-09	1.4	1.20E-02	2
IL18R1	2.30E-10	-1.5	2.40E-01	-2.1
IMPA2	**1.70E-05**	**-2.4**	**3.00E-02**	**-1.6**
KDM6A	3.40E-07	-1.4	5.70E-01	-6.3
KLRA2	9.80E-09	1.7	4.20E-01	2.1
KLRD1	2.50E-05	-1.3	4.10E-01	-2.1
KRT23	1.60E-04	1.5	3.30E-01	2.8
LAMC1	9.50E-10	-1.4	1.70E-03	-3.8
NFIA	7.90E-12	-1.6	9.40E-02	-2.2
NPL	1.00E-03	1.3	3.10E-01	2.3
PF4	5.90E-02	1.2	6.10E-02	4.9
PLCB1	1.60E-10	-1.7	4.60E-02	-2.7
PPP1R1B	1.20E-02	1.4	4.80E-01	2.1
PROK2	2.80E-10	-1.4	2.40E-01	-2.8
PTX3	1.50E-05	-1.5	1.30E-01	-7.2
SERPINB2	1.40E-08	1.6	3.40E-01	2.4
TAC3	1.50E-05	1.7	3.40E-01	3.4
TAF13	1.10E-08	1.9	6.80E-02	2.1
TGM3	1.40E-06	-1.5	2.30E-01	-2.9
TSPY1	5.30E-11	-1.5	3.70E-01	-3.2
USP18	2.40E-08	1.6	1.10E-01	2.1
VDR	5.90E-10	-1.4	1.20E-01	-2.2

* The data is presented with p-value and fold change (FC) for each gene as well as gene symbol.

4.3.2.3   Selection of genes associated with key pressure points (cattle)

A pressure point is defined as times of immune/stress challenge with potential welfare impact. At the outset of the project, We hypotheses that handling, transport, adaptation of feed or movement of animals resulting in changes to social structure are key pressure points with potential to impact upon the eventual 'fit for purpose' status. Meta-analysis of public sourced transcriptomic databases combined with transcriptomic data sourced from cattle undergoing production stressors enabled in an depth comparison between the following stressors: adaptation of food source (from pasture based grass-fed to feedlot or pen based pellet/high energy feed or silage feed), cold and psychological stress and social stress (transition from open paddock to intensive close-contact pens and introduction of new pen mates) and farm of origin. Genes associated with pressure points have been identified by cross comparison of genes overrepresented in animals exposed to these stress variables with production outcomes (ADG, MSA index score and disease status). Comparison analysis has revealed that the point of most stress is in relation to rumen adaptation to a new feed source and In particular, transition from a primarily pasture fed diet to a finishing diet rich in pellets/chaff and other feeds designed to optimise high energy conversely trigger the expression of over 7000 differentially regulated genes (data not shown). The pathways associated with these genes in samples taken during the period of transition are largely related to metabolism (**Table 4.3.9.**) and although there is considerable overlap with pathways highlighted in those cattle capable of achieving high ADG, the predicted activation or inhibition is primarily in opposition to a beneficial outcome. This is highlighted in Figure 4.3.7 where it is clear that exposure of cattle to feed other than grass results in activation of cholesterol biosynthesis, lipogenesis, the immune response, and cholesterol efflux. The potential effect of this response is made clear in Figure 4.3.8, showing the effect of genes expressed in a cohort of cattle suffering from acidosis on the same pathway. This suggests that transition of cattle from pasture to a feedlot/high energy feed diet results in an almost identical response as those animals with acidosis. In stark opposition to both the previous figures, Figure 4.3.9 shows the response of cattle capable of achieving high ADG. It was of particular interest to note that feeding cattle with silage (Figure 4.3.10) supressed the immune response however similar cholesterol activity was observed as per grain/high energy or acidosis cattle.

Table 4.3.9. Biological pathways identified in cattle transitioned to a diet comprising of pellets/chaff/high energy feed or silage feed in comparison to pasture fed cattle. generated using Partek Pathway function within Partek Genomics Suite (Partek Inc).

| Pathway Name	Enrichment Score	Enrichment p-value	# genes in list
Metabolic pathways	22.1	2.5E-10	431
Thermogenesis	12.8	2.9E-06	102
MAPK signaling pathway	5.9	2.6E-03	102
Endocytosis	9.5	7.6E-05	92
Focal adhesion	10.1	4.3E-05	78
Rap1 signaling pathway	5.6	3.7E-03	75
Purine metabolism	11.8	7.7E-06	72
cGMP-PKG signaling pathway	10.3	3.3E-05	67
Cellular senescence	9.2	1.0E-04	67
Regulation of actin cytoskeleton	4.0	1.8E-02	66
Carbon metabolism	24.8	1.6E-11	64
Oxidative phosphorylation	9.4	8.5E-05	63
Protein processing in endoplasmic reticulum	7.4	6.0E-04	63
AMPK signaling pathway	16.6	6.1E-08	62
Ubiquitin mediated proteolysis	15.1	2.8E-07	62
Autophagy - animal	14.4	5.3E-07	61
Chemokine signaling pathway	3.7	2.5E-02	61
Insulin signaling pathway	13.2	1.8E-06	59
Adrenergic signaling in cardiomyocytes	10.8	2.1E-05	57
Fluid shear stress and atherosclerosis	5.8	3.0E-03	57
Hepatocellular carcinoma	3.7	2.5E-02	55
Axon guidance	3.6	2.6E-02	54
Tight junction	3.2	4.0E-02	54
Apelin signaling pathway	8.3	2.4E-04	53
FoxO signaling pathway	7.8	3.9E-04	53
RNA transport	3.3	3.8E-02	53
Oxytocin signaling pathway	4.4	1.2E-02	52
Retrograde endocannabinoid signaling	4.0	1.8E-02	52
Insulin resistance	11.9	6.9E-06	50
Glucagon signaling pathway	16.1	9.7E-08	49
Platelet activation	8.1	3.1E-04	49
Hippo signaling pathway	4.6	1.0E-02	49
Osteoclast differentiation	3.6	2.6E-02	48
AGE-RAGE signaling pathway	8.1	3.0E-04	47
TNF signaling pathway	6.3	1.8E-03	45
Sphingolipid signaling pathway	4.8	7.9E-03	45
Relaxin signaling pathway	3.9	2.0E-02	45
Wnt signaling pathway	3.4	3.5E-02	45
Toxoplasmosis	3.2	4.2E-02	45
Neurotrophin signaling pathway	4.9	7.2E-03	44
HIF-1 signaling pathway	7.0	9.2E-04	42
Thyroid hormone signaling pathway	4.8	8.4E-03	42
Leukocyte transendothelial migration	5.7	3.3E-03	41

Pathway Name	Enrichment Score	Enrichment p-value	# genes in list
Dopaminergic synapse	3.0	5.2E-02	41
Biosynthesis of amino acids	18.0	1.5E-08	40
Fc gamma R-mediated phagocytosis	7.0	8.8E-04	40
Pyrimidine metabolism	6.7	1.2E-03	39
Longevity regulating pathway	7.3	6.9E-04	38
Melanogenesis	5.9	2.8E-03	38
Oocyte meiosis	3.9	2.1E-02	38
RNA degradation	11.0	1.7E-05	36
Glycolysis / Gluconeogenesis	13.3	1.7E-06	34
GnRH signaling pathway	4.7	9.4E-03	34
Aldosterone synthesis and secretion	3.3	3.8E-02	33
Cardiac muscle contraction	5.3	5.1E-03	32
Mitophagy - animal	8.6	1.8E-04	31
p53 signaling pathway	6.4	1.7E-03	31
mRNA surveillance pathway	3.7	2.5E-02	31
Progesterone-mediated oocyte maturation	3.1	4.5E-02	31
Adipocytokine signaling pathway	5.1	5.9E-03	30
ECM-receptor interaction	4.2	1.5E-02	30
Peroxisome	4.1	1.7E-02	30
PPAR signaling pathway	3.9	2.0E-02	30
Glioma	4.6	9.6E-03	29
Renal cell carcinoma	4.2	1.5E-02	29
Proteasome	15.9	1.3E-07	27
Acute myeloid leukemia	4.2	1.5E-02	27
Bacterial invasion of epithelial cells	4.0	1.8E-02	27
Arginine and proline metabolism	7.7	4.7E-04	26
Regulation of lipolysis in adipocytes	6.4	1.6E-03	26
Longevity regulating pathway - multiple species	3.6	2.7E-02	26
Citrate cycle (TCA cycle)	17.4	2.7E-08	25
Pyruvate metabolism	15.5	1.8E-07	25
Adherens junction	3.1	4.7E-02	25
Amphetamine addiction	3.6	2.8E-02	24
Renin secretion	3.0	4.8E-02	24
VEGF signaling pathway	3.1	4.3E-02	23
Glutathione metabolism	3.1	4.6E-02	20
Fatty acid metabolism	3.1	4.6E-02	19
Basal transcription factors	5.0	7.0E-03	18
Hedgehog signaling pathway	4.3	1.4E-02	18
Notch signaling pathway	4.1	1.7E-02	18
Propanoate metabolism	5.3	5.0E-03	17
Cysteine and methionine metabolism	3.9	2.0E-02	17
RNA polymerase	7.0	8.7E-04	16
Glycine, serine and threonine metabolism	4.0	1.9E-02	16

Pathway Name	Enrichment Score	Enrichment p-value	# genes in list
2-Oxocarboxylic acid metabolism	13.4	1.5E-06	15
Pentose phosphate pathway	6.6	1.4E-03	15
Fructose and mannose metabolism	5.0	6.7E-03	15
Autophagy - other	4.7	8.8E-03	15
Apoptosis - multiple species	3.2	3.9E-02	14
Nicotinate and nicotinamide metabolism	3.2	3.9E-02	14
Starch and sucrose metabolism	3.8	2.3E-02	12
Glyoxylate and dicarboxylate metabolism	3.5	3.0E-02	12
Arginine biosynthesis	3.8	2.1E-02	11
Histidine metabolism	3.6	2.8E-02	11
Nitrogen metabolism	4.5	1.1E-02	9
Valine, leucine and isoleucine biosynthesis	3.6	2.8E-02	3
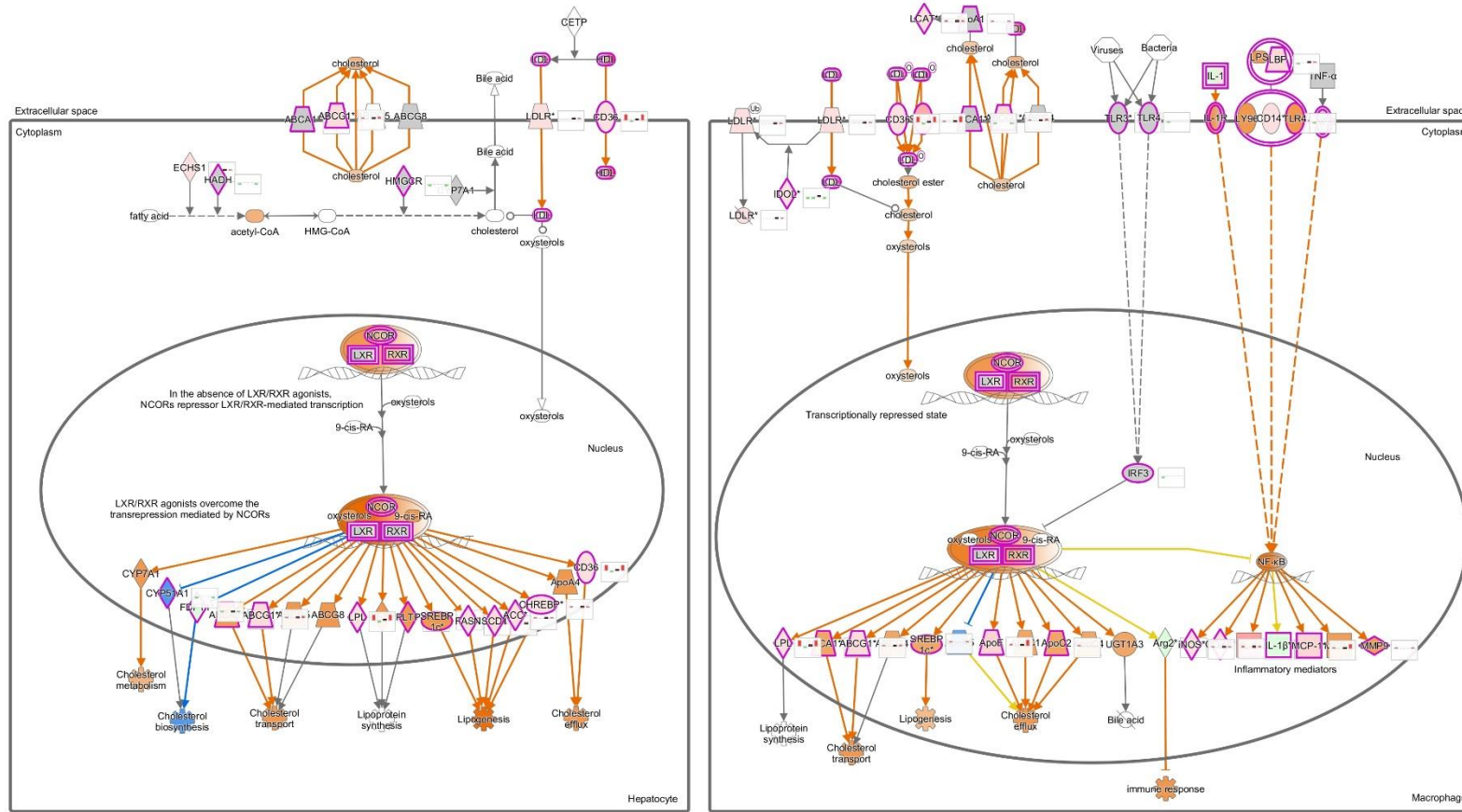
Figure 4.3.7. Inhibition of Cholesterol biosynthesis and immune mechanisms in cattle in cattle transitioned to a diet comprising of pellets/chaff/high energy feed in comparison to pasture fed cattle. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.
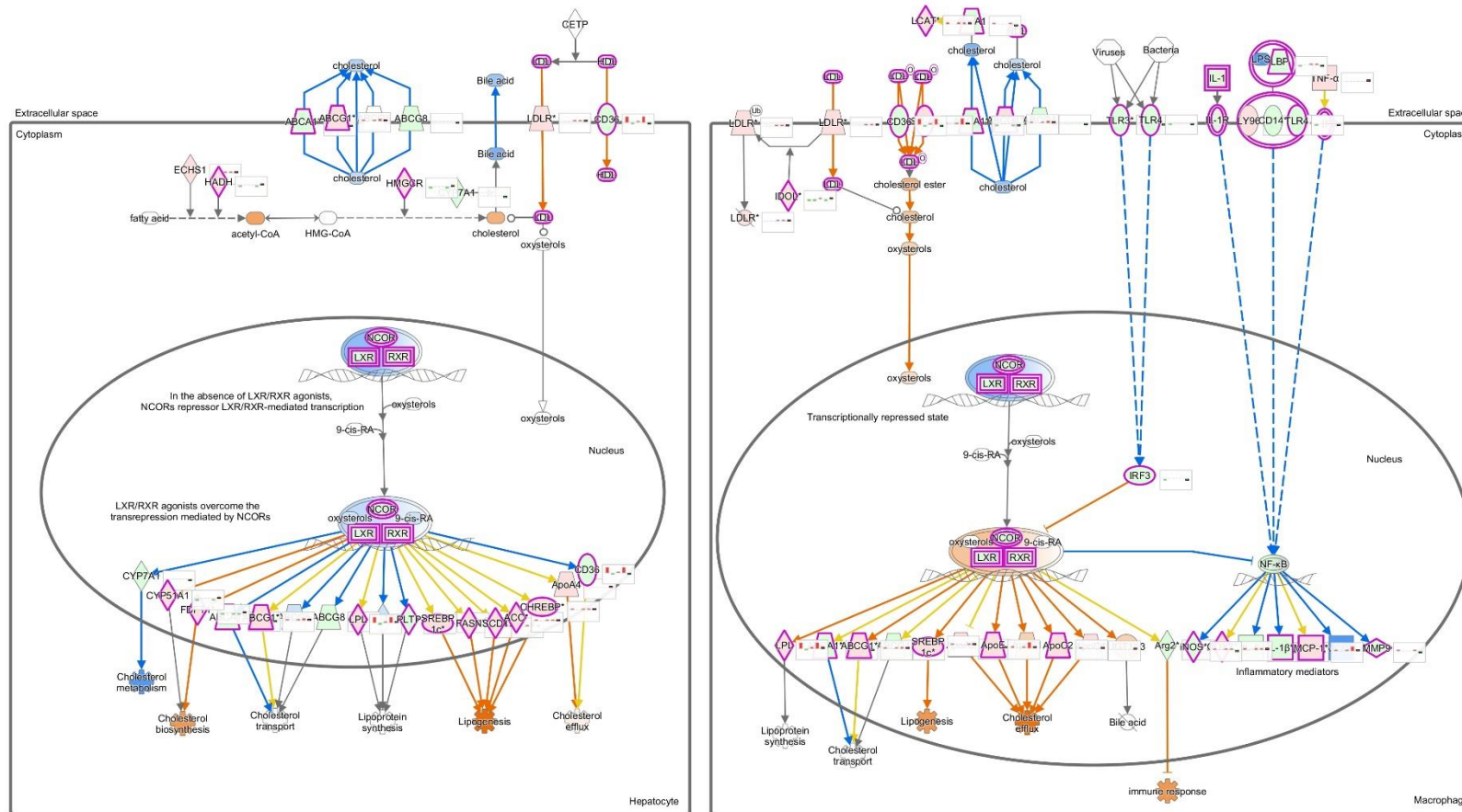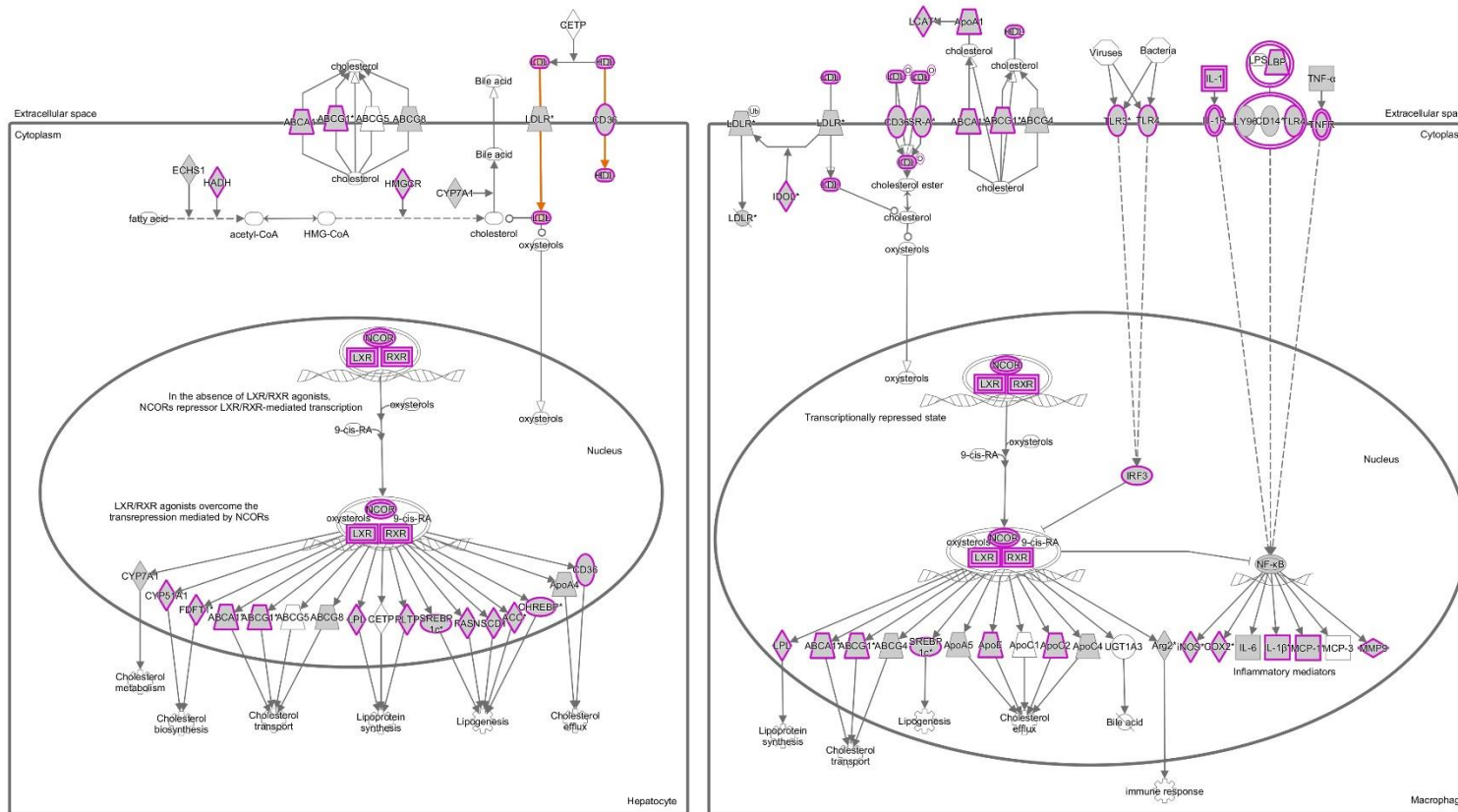
Figure 4.3.8. Inhibition of Cholesterol biosynthesis and immune mechanisms in cattle suffering from active acidosis in comparison to grassfed cattle. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.
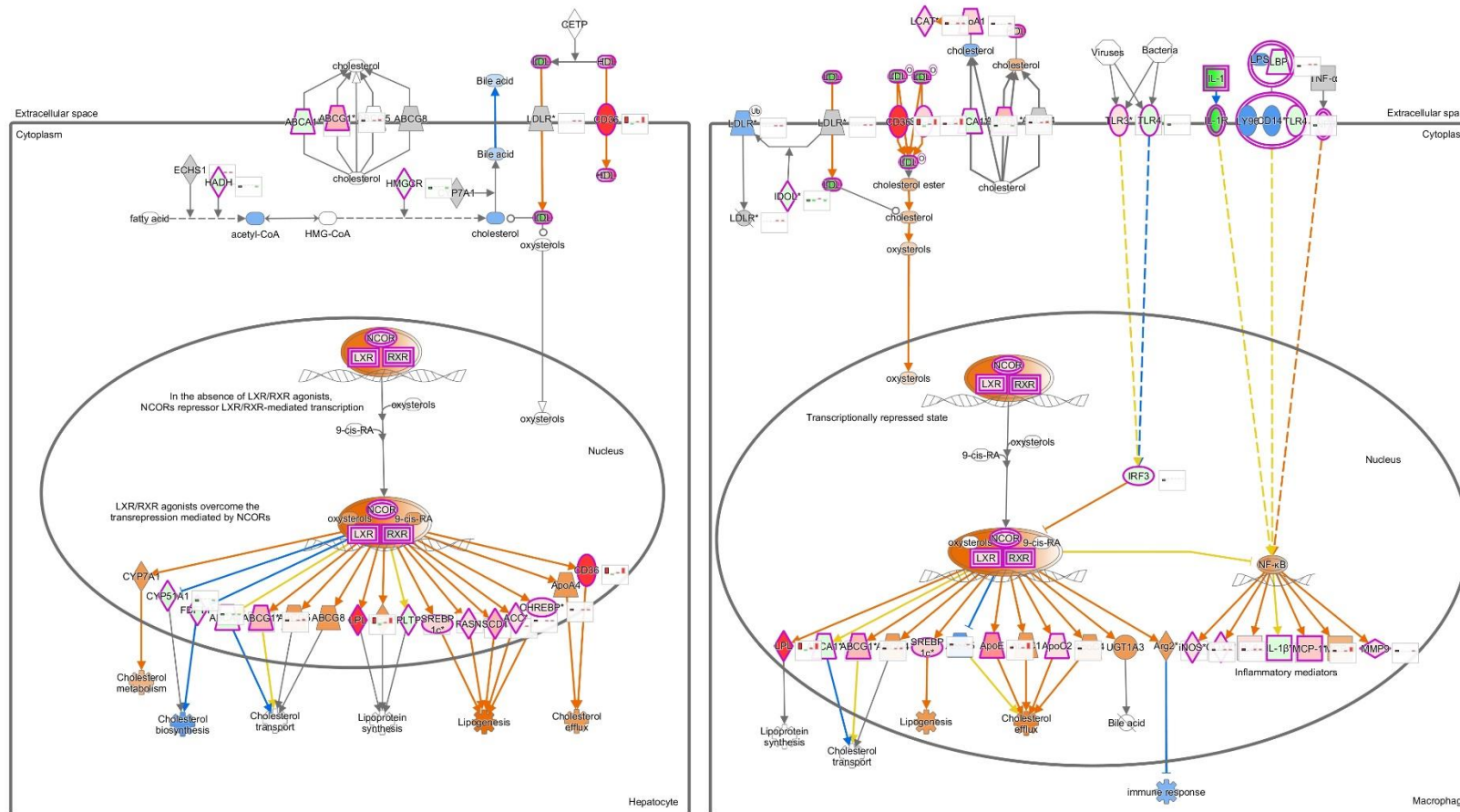
Figure 4.3.9. Cattle capable of achieving high ADG show no activation of Inhibition of Cholesterol biosynthesis and immune. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.

Figure 4.3.10. Inhibition of Cholesterol biosynthesis and immune mechanisms in cattle in cattle transitioned to a diet comprising of silage feed in comparison to pasture fed cattle. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree

4.3.2.4 Selection of gene expression correlates of fitness (Sheep)

Three datasets were utilised to identify genes whose expression are putative correlates of fitness in the ovine model (Table 4.3.10); the samples were obtained from sheep exposed to either *Brucella ovis* or MAP and comprising of 50 control and 144 pathogen exposed sheep.

Following individual normalisation, gene lists were collated to identify differentially expressed genes common between disease models and regulated in the resilient cohort of sheep in comparison with the control and susceptible sheep. This resulted in 1437 (**Appendix 8.4**) differentially regulated genes (807 downregulated and 630 upregulated). Of these, 30 genes of interest whose expression are significantly regulated in sheep achieving resilience to disease are listed in Table 4.3.9. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**. Genes previously validated as markers of disease exposure in MAP exposed Merino sheep (P.PSH.0311 or P.PSH.0576 final report) are *italicised*.

Table 4.3.10. Differentially upregulated and downregulated genes expressed in sheep achieving resilience to disease. Genes selected for qPCR validation for putative markers of Immune Fitness are highlighted **bold**. Genes previously validated as markers of disease exposure in MAP exposed Merino sheep (P.PSH.0311 or 0576 final report) are *italicised*.

Transcript ID	Entrez ID	Gene Symbol	p-value	Fold-Change
Bt.23178.1.S1_at	280760	DCN	7.63E-07	-6.5
Bt.3595.1.S1_at	282660	MGP	1.18E-09	-4.9
Bt.15972.1.S1_at	281193	GJA1	1.17E-07	-4.7
Bt.6338.1.S1_at	616981	FERMT2	8.94E-08	-4.7
Bt.3478.1.A1_s_at	281040	CAV1	2.35E-07	-4.2
Bt.27127.1.A1_at	---	---	3.09E-07	-4.2
Bt.12825.1.S1_at	515610	ACTA2	1.07E-08	-4.1
Bt.8124.1.S1_at	282188	COL1A2	3.49E-07	-3.8
Bt.2506.1.S1_at	506627	DKK3	1.66E-07	-3.7
Bt.11424.1.S1_at	---	---	2.07E-08	-3.7
Bt.4903.1.S2_at	281945	NR2F2	6.22E-08	-3.6
Bt.23583.1.S1_at	**378475**	**IL21**	**4.62E-06**	**-2.6**
Bt.3046.2.S1_at	**497016**	**FCN1**	**0.001239**	**-2.5**
Bt.24933.1.S1_at	**510102**	**RARRES1**	**0.000234**	**-2.4**
Bt.15952.1.A1_at	*789054*	*SESTD1*	*3.32E-10*	*-1.6*
Bt.13273.1.S1_at	*338437*	*SOCS2*	*1.19E-06*	*-1.6*
Bt.22434.1.S1_at	**1.05E+08**	**ZFP75A**	**5.11E-06**	**2.2**
Bt.5304.1.S1_at	**615507**	**GSTM3**	**4.62E-06**	**2.4**
Bt.22867.1.S1_x_at	282534	BOLA-DQA1	2.93E-07	2.8
Bt.209.3.S1_at	777776	LYZ	6.72E-06	2.9
Bt.351.1.S1_at	282196	CORO1A	1.49E-06	3.0
Bt.18674.1.A1_at	783269	BCL11B	5.71E-07	3.1

Transcript ID	Entrez ID	Gene Symbol	p-value	Fold-Change
Bt.22021.1.S1_at	506759	IFI16	1.91E-06	3.1
Bt.13974.1.A1_at	1E+08	BANK1	1.43E-06	3.3
Bt.28518.1.S1_s_at	404103	PTI	8.82E-07	3.4
Bt.21431.1.S1_at	529757	LTB	5.65E-07	3.5
Bt.11581.1.S1_at	507790	PF4	1.02E-06	3.6
Bt.28732.1.S1_at	407199	TRD@	2.55E-08	3.8
Bt.15912.1.S1_at	767910	PLAC8	7.42E-07	3.9
Bt.29824.1.S1_s_at	**515712**	**BOLA**	**0.000127**	**4.9**

* The data is presented with p-value and fold change for each gene as well as transcript ID, Entrez number and gene symbol.

These genes are presented as potential candidates of immune fitness in the sheep model, however additional validation will be required to narrow-down to a practicable yet effective number of genes to include.

4.3.2.5 Pathway analysis of genes of interest (cattle)

Pathway analysis seeks to cluster genes to functional groups and then finding overrepresented groups in filtered gene lists using a Fisher's test. Significant pathways are those with an enrichment score ≥ 3 or p-value ≤0.05 and 38 significant key canonical pathways were associated with the differential expressed genes in cattle that achieved resilience to disease following exposure (Table 4.3.11) or achieved a high average daily gain or high MSA index score (Table 4.3.12).

Table 4.3.11. Biological pathways identified in disease resilient cattle following exposure to disease causing pathogens including *Mycobacterium bovis*, *Mycobacterium avium* subsp. *paratuberculosis*, *Rhipicephalus microplus*, Pestivirus and *Neotyphodium coenophialum*, generated using Partek Pathway function within Partek Genomics Suite (Partek Inc).

Pathway Name	Enrichment Score*	Enrichment p-value	# genes in list
Phagosome	15.8	1.32E-07	10
Cell adhesion molecules (CAMs)	15.6	1.66E-07	9
Leishmaniasis	19.3	4.20E-09	9
Th17 cell differentiation	15.1	2.67E-07	8
Th1 and Th2 cell differentiation	15.8	1.31E-07	8
Antigen processing and presentation	15.9	1.24E-07	8
Allograft rejection	17.9	1.73E-08	8
Graft-versus-host disease	20.1	1.91E-09	8
Hematopoietic cell lineage	11.8	7.70E-06	7
Intestinal immune network for IgA production	12.5	3.67E-06	6
Hepatocellular carcinoma	4.4	1.18E-02	4
Apoptosis	4.8	7.99E-03	4

Pathway Name	Enrichment Score*	Enrichment p-value	# genes in list
Glutathione metabolism	8.6	1.83E-04	4
Oxytocin signaling pathway	3.1	4.60E-02	3
Carbon metabolism	3.8	2.17E-02	3
IL-17 signaling pathway	3.9	2.05E-02	3
Endocrine resistance	4.1	1.61E-02	3
Drug metabolism - other enzymes	5.0	6.53E-03	3
Drug metabolism - cytochrome P450	5.8	3.00E-03	3
Pantothenate and CoA biosynthesis	9.2	1.01E-04	3

* Significant pathways are those with an enrichment score ≥ 3 or p-value ≤0.05.

Table 4.3.12. Biological pathways identified in cattle achieving high ADG and high MSA index scores generated using Partek Pathway function within Partek Genomics Suite (Partek Inc).

Pathway Name	Enrichment Score*	Enrichment p-value	# genes in list
Cytokine-cytokine receptor interaction	3.9	2.1E-02	13
Endocytosis	3.2	3.9E-02	11
Chemokine signaling pathway	3.2	4.0E-02	9
Cellular senescence	4.2	1.6E-02	9
Cell cycle	3.5	3.1E-02	7
Hematopoietic cell lineage	4.3	1.4E-02	7
Antigen processing and presentation	5.9	2.8E-03	7
Biosynthesis of amino acids	4.7	8.9E-03	6
VEGF signaling pathway	3.1	4.7E-02	4
Arginine and proline metabolism	3.4	3.3E-02	4
beta-Alanine metabolism	3.0	5.4E-02	3
DNA replication	3.0	5.4E-02	3
Circadian rhythm	3.4	3.3E-02	3
Th17 cell differentiation	3.5	8.9E-03	5
Th1 and Th2 cell differentiation	5.2	2.1E-06	4

* Significant pathways are those with an enrichment score ≥ 3 or p-value ≤0.05.

This study identified differential expression of genes between high and low ADG achieving cattle and those with resilience to disease. Several common pathways were identified between these two production outcomes including focus genes attributed to antigen presentation pathways. Cattle resilient to disease expressed genes predicted to inhibit MHC class II Antigen presenting pathway and activation of the MHC class I pathway (Fig. 4.3.11) whereas cattle with high ADG express genes associated with activation of the MHC class II Antigen presenting pathway and inhibition of the MHC

class I pathway (see **Appendix 8.4**). IFN-γ was a predicted upstream regulator in both the disease resilient (downregulated) and the high ADG cohort (upregulated). Upregulation of IFN-γ resulted in a predicted activation of the MHC class II antigen presentation pathway, and inhibition of the MHC class I antigen presentation pathway (see **Appendix 8.4**). MHC class II presentation displays antigen fragments to CD4^{+} T lymphocytes, directing a T cell response; either Th1 or Th2 focused dependent on the pattern of cytokine activation. Fonseca (Fonseca et al., 2019) identified antigen presentation as a key pathway associated with feed efficiency. Enhanced antigen presentation may suggest a rapid immune response to pathogens or disease processes before they negatively impact on health and performance of the individual. Decreased antigen presentation in low ADG cattle may prevent a strong immune response occurring, leading to insidious or subclinical disease processes preventing maximal weight gain.
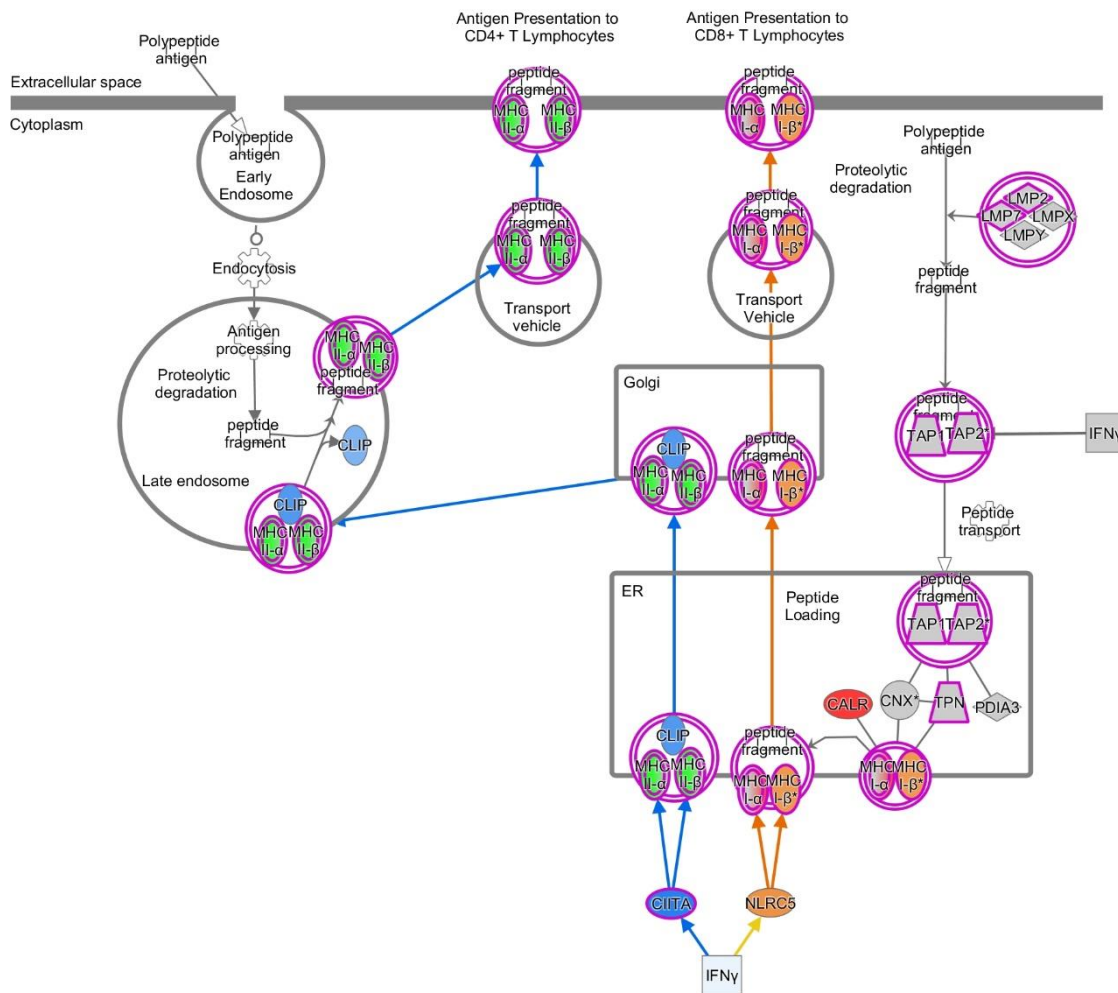
Figure 4.3.11. Inhibition of MHC class II Antigen presenting pathway and activation of MHC class I pathway in disease and stress resilient cattle. Red = array measured up-regulated gene expression, green = array

measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.

Following antigen presentation through MHC class II to an immature CD4^{+} T cell, the pattern of interleukin and cytokine production will direct differentiation of the CD4^{+} T lymphocytes down a Th1 or Th2 cell response. Th1 and Th2 activation pathways were overrepresented in both the disease resilient and the high ADG cohort, the gene expression patterns predict inhibition of a Th1 cell mediated immune response in both the disease resilient (Fig. 4.3.12) and high weight gain cohort (figure not shown). Activation of Th1 and Th2 lineages direct the immune system towards cell mediated immunity and antibody mediated immunity respectively. The immune mediators IL-10, IFN-γ, and IL-4 were key upregulated genes directing a Th2 response. IL-27, ICAM1, CD86, CD3, ICAM1, IL-12R, CXCR3, TNF-β, Galectin-9 were also upregulated. Key upregulated genes in Th1 activation pathways included CD86, ICAM1, CD3, IL4R, IL2R, Tslpr, IL-10, IFN-γ, IL-12R.

As visualized in Fig. 4.3.12, both Th1 and Th2 cell activation is overall inhibited despite activation of genes within these pathways. Th2 cell activation is inhibited more than Th1. That is, although both Th1 and Th2 cell activation are inhibited, Th1 is inhibited to a lesser degree than Th2, swaying the immune response towards Th1 functions. Low level activation of the Th1 pathway may indicate a functional and actively surveying immune system ready to react if a pathogen was detected, but not requiring excessive energy. An 'on alert' monitoring immune system opposed to an active system may favour high ADG through a healthy individual with rapid immune responses. This hypothesis is plausible as an overactive immune system or actively infected animal will experience decrease growth as energy is prioritised for immune function not weight gain (Fonseca et al., 2019).

IL-10 was significantly upregulated molecule that IPA analysis identified. IL10 is an immune regulatory cytokine with anti-inflammatory functions. IL10 targets antigen presenting cells and inhibits their release of proinflammatory cytokines including IL-1 alpha, IL-1 beta, macrophage colony stimulating factor, granulocyte colony-stimulating factor, IL-6, IL-8 and TNF-alpha. Upregulation of IL-10 in high ADG individuals indicates an anti-inflammatory environment preserving energy for growth and may be representative of hardy disease-free individuals.

Of significance is the finding that cattle achieving resilience of disease or those gaining high ADG or MSA scores commonly express genes associated with metabolism.

Figure 4.3.12. Inhibition of a Th1 cell mediated immune response and activation of Th2 cell mediated immune response in disease and stress resilient cattle. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.

4.3.2.6   Pathway analysis of genes of interest (sheep)

Pathway analysis seeks to identify genes to groups and then finding overrepresented groups in filtered gene lists using a Fisher's test. Significant pathways are those with an enrichment score ≥ 3 or p-value ≤0.05 and 38 significant key canonical pathways were associated with the differential expressed genes in sheep that achieved resilience to disease following exposure (Table 4.3.13). Although not all the same genes were differentially regulated, the focus genes overrepresented within the resilient sheep cohort display an overlap to the Th1 and Th2 pathway highlighted in the cattle cohorts with predicted inhibition of both Th1 cell and Th2 mediated immune responses (Fig. 4.3.13) and in contrast, in the sheep model there is predicted enhanced activation of both MHC class I and MHC class II pathways (Fig. 4.3.14).

Table 4.3.13. Biological pathways identified in disease resilient sheep following exposure to *Brucella ovis* or MAP, generated using Partek Pathway function within Partek Genomics Suite (Partek Inc).
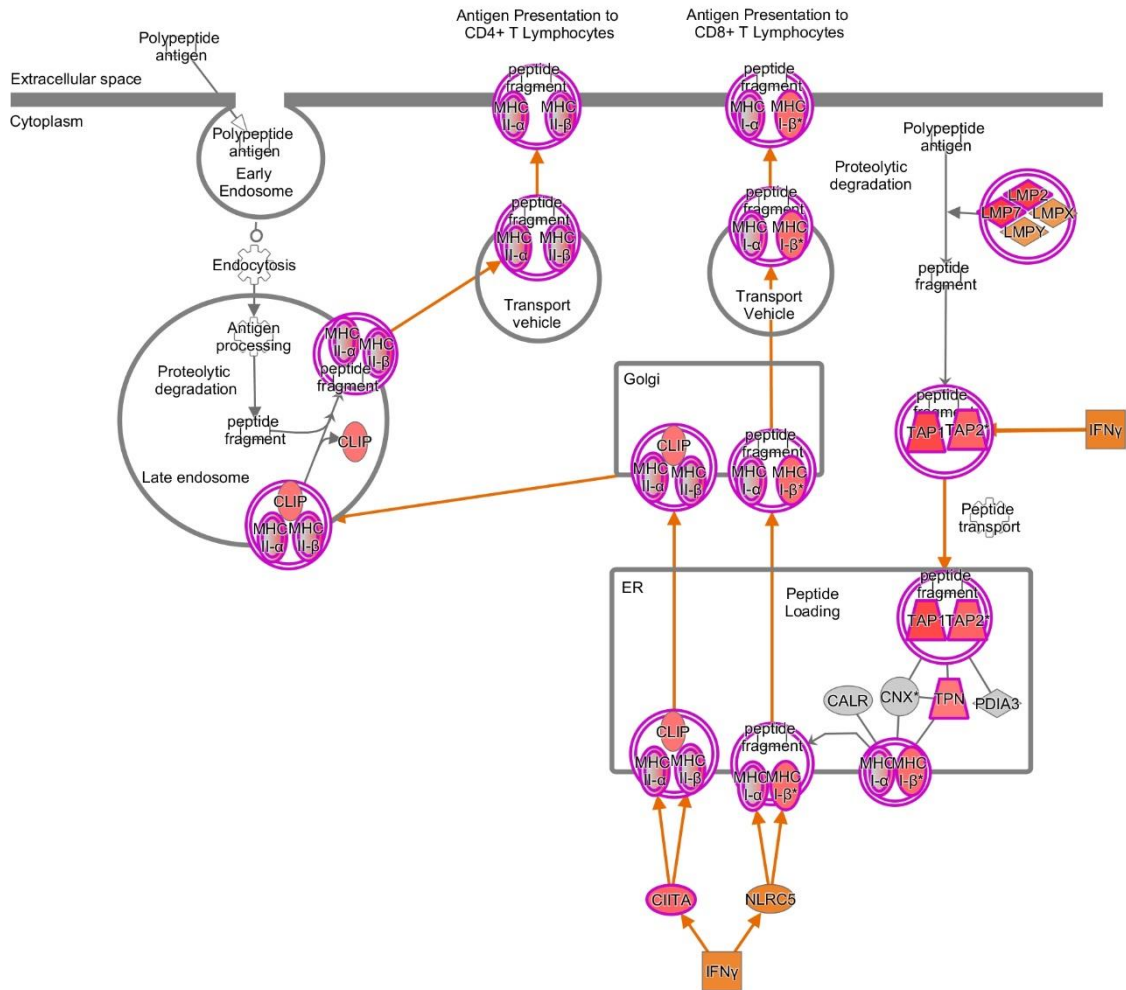
Pathway Name	Enrichment Score*	Enrichment p-value	# of genes differentially expressed
PI3K-Akt signaling pathway	3.6	2.7E-02	33
Cell adhesion molecules (CAMs)	20.4	1.4E-09	30
Focal adhesion	10.2	3.9E-05	29
MAPK signaling pathway	4.1	1.6E-02	28
Rap1 signaling pathway	7.2	7.8E-04	25
Regulation of actin cytoskeleton	7.4	5.9E-04	25
Chemokine signaling pathway	8.2	2.9E-04	25
Ras signaling pathway	5.4	4.3E-03	24
Phagosome	7.8	4.0E-04	23
Hematopoietic cell lineage	15.8	1.4E-07	22
Leukocyte transendothelial migration	12.4	4.3E-06	21
Fc gamma R-mediated phagocytosis	15.4	2.0E-07	21
Axon guidance	5.8	3.1E-03	20
Th17 cell differentiation	8.6	1.9E-04	19
Platelet activation	9.2	1.0E-04	19
Viral myocarditis	19.3	4.1E-09	19
Antigen processing and presentation	14.8	3.6E-07	18
Hippo signaling pathway	4.9	7.7E-03	17
Th1 and Th2 cell differentiation	8.8	1.4E-04	17
ECM-receptor interaction	11.1	1.6E-05	17
Jak-STAT signaling pathway	3.1	4.4E-02	16
T cell receptor signaling pathway	6.7	1.2E-03	16
Natural killer cell mediated cytotoxicity	7.1	8.2E-04	16
Wnt signaling pathway	4.1	1.7E-02	15
Intestinal immune network for IgA production	14.9	3.5E-07	15
Protein digestion and absorption	4.4	1.2E-02	14
Allograft rejection	14.8	3.8E-07	14
B cell receptor signaling pathway	6.2	2.1E-03	13
Adherens junction	8.2	2.9E-04	13
Graft-versus-host disease	14.4	5.8E-07	13
Primary immunodeficiency	17.8	1.8E-08	13
NF-kappa B signaling pathway	4.2	1.5E-02	12
Staphylococcus aureus infection	8.0	3.5E-04	12
Bacterial invasion of epithelial cells	4.5	1.1E-02	11
Complement and coagulation cascades	3.2	4.2E-02	10
Fc epsilon RI signaling pathway	3.3	3.5E-02	9
Longevity regulating pathway - multiple species	3.2	4.3E-02	8
Hippo signaling pathway - multiple species	3.4	3.2E-02	5

* Significant pathways are those with an enrichment score ≥ 3 or p-value ≤0.05.

Figure 4.3.13. Inhibition of both Th1 and Th2 cell mediated immune responses in disease resilient sheep. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.

Figure 4.3.14. Activation of both MHC class II Antigen presenting pathway and MHC class I pathway in disease resilient sheep. Red = array measured up-regulated gene expression, green = array measured down-regulated gene expression, orange = predicted activation, blue = predicted inhibition. A lighter shade within those colours indicates activation/inhibition/up or downregulation to a lesser degree.

4.3.3   qRT-PCR validation

Validation of selected genes was performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The selected genes were those whose expression was predicted in the analysis of transcriptomic data and especially that of the gene based correlates of immune fitness predicted following analysis of externally sourced datasets utilising the RNA collected from pathogen (MAP, *H.contortus* or *Salmonella)* or production stressor (feedlot and farm) exposed sheep and cattle serves to validate the model. Validation of the selected genes lends weight to selection of other genes within the gene lists.

For the cattle the genes GZMB, KLRC1, IMPA2, CSF1, HSPB1 and PTGR1 were selected for validation. Validation of disease expression was performed on samples sourced from MAP exposed cattle (3.1.7), the meta-analysis predicted expression of disease resilience was verified for the genes GZMB and KLRC1in MAP resilient calves in samples collected at 9 months of age and again at 16 months. The expression of the genes IMPA2, CSF1, HSPB1 and PTGR1 was validated in either the early (9 months) or the late (16 months) timepoint. Expression of the genes GZMB, KLRC1, IMPA2, CSF1 and HSPB1 was validated to be relevant to identifying cattle from the feedlot trial (Section 3.1.6) capable of achieving a high ADG in samples obtained and similarly the predicted expression for animals capable of achieving a high MSA index score was verified in the feedlot cattle for the genes GZMB, KLRC1, CSF1 and HSPB1 (Table 4.3.14).

Validation for sheep (Table 4.3.15) included the genes BOLA1a, FCN1/2, GSTM3, ZFP75A, RARRES1, TNFα, IL-1α and IL-21 as markers of immune fitness to disease as predicted in the meta-analysis. TNFα and IL-1α were selected as they were strongly predicted upstream regulators of many of the predicted genes of interest. The transcriptomic predicted expression was validated in samples obtained from MAP and or *H.contortus* exposed and control animals for the genes BOLA1a, FCN1/2, GSTM3, ZFP75A, RARRES1, TNFα, IL-1α and IL-21. The predicted expression of the genes BOLA1a, FCN1/2, GSTM3, TNFα and IL-1α was confirmed in a cohort of sheep experimentally exposed to *Salmonella*. The genes FCM1/2, RARRES1, TNFα, IL-1α and IL-21 were also associated to the potential to gain weight (high ADG) as determined by previously reported (P.PSH.0311 and P.PSH.0576) transcriptomic analysis of the MAP exposed sheep datasets, taking into account weight gain statistics of MAP exposed and control sheep (Section 3.1.4 and 3.1.5).

The majority of the selected genes were demonstrated to be suitable candidates as markers of immune fitness in terms of disease resilience or potential to gain weight.

Table 4.3.14. qRT-PCR validation of selected potential correlates of fitness (cattle). Where expression matched that predicted within the appropriate transcriptomic dataset, the fold change is **bold**.

Variable tested	Timepoint tested	qRT-PCR fold change					
		GZMB	KLRC1	IMPA2	CSF1	HSPB1	PTGR1
Potential to gain weight (ADG)	Induction (feedlot)	**4.4**	**-1.5**	**-8.01**	-2.3	**2.6**	
	Post induction (feedlot)	**1.9**	**-1.5**	**-2.18**	**3.61**	**1.4**	
Potential to achieve high MSA score	Induction (feedlot)	**-1.5**	**-1.6**		**-1.5**	**-15.3**	
	Post induction (feedlot)	**-1.7**	**-2.5**		**-23.7**	**-2.0**	
Potential to detect disease (MAP)	Resilient cattle (9 months)	**3.5**	**2.0**	**-1.9**	**-5.9**	**-3.2**	
	Resilient cattle (16 months)	**2.4**	**3.2**				**-2.0**

P value for all data ≤0.005.

Table 4.3.15. qRT-PCR validation of selected potential correlates of fitness (sheep). Where expression matched that predicted within the appropriate transcriptomic dataset, the fold change is **bold**.

Variable tested	Pathogen exposure	qRT-PCR Fold Change							
		BOLA 1a	FCN1/2	GSTM3	ZFP75A	RARRES1	TNFa	IL-1a	IL-21
Resilience to disease	MAP & HC	**1.4**	**-2.2**	**1.5**	**1.9**	**-2.1**	**-1.7**	**-1.8**	**-4.1**
	Salmonella	**2.0**	**-2.1**	**2.8**		10.6	**-1.6**	**-2.1**	
Capacity to achieve high ADG	MAP & HC resilient & control	-2.9	1.7		-4	**-1.8**	**-1.6**	**-2.1**	**-2.1**

P value for all data ≤0.005.

### 4.3.4   Assessment of telomere length following exposure to production stress

4.3.4.1   Relative telomere length and immune fitness in sheep

The telomere qPCR assay for sheep was optimised for the instrument conditions and master mix used. A standard curve for the optimised assay is shown in Fig. 4.3.15, using genomic DNA from a Merino sheep. The Telomere qPCR experiments had an average efficiency score of 105 % with tel1b/2b primers with sample amplification early in the thermal cycling (average Ct values of 18.7), compared to the single copy reference gene amplification (average Ct 27.0).
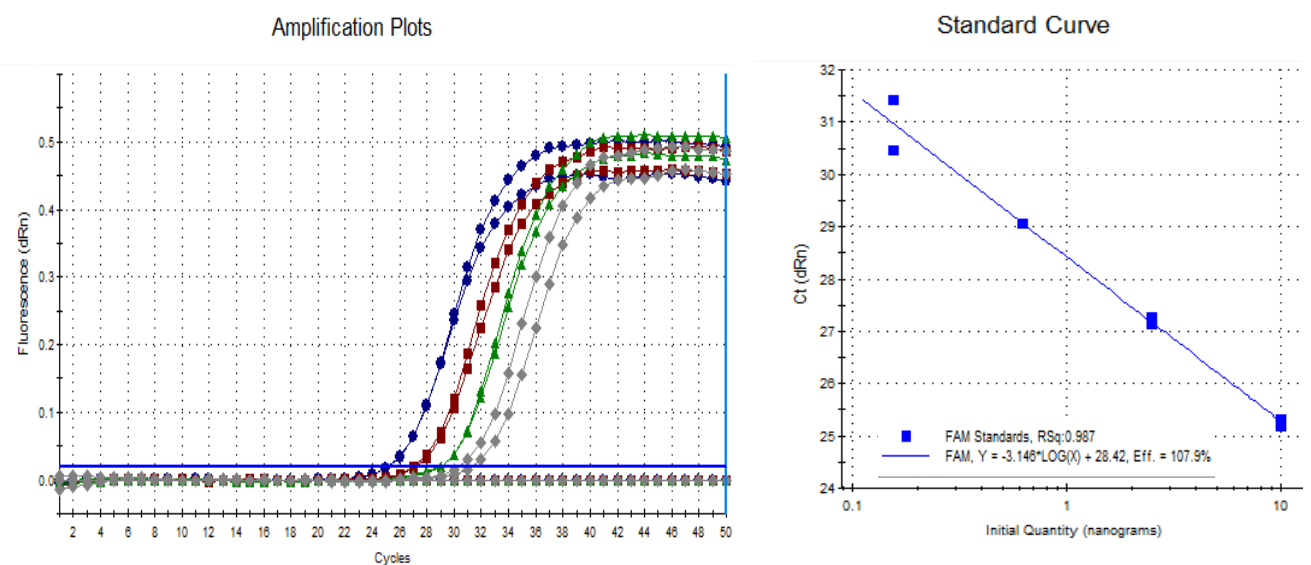
Figure 4.3.15. Amplification plots and Standard curve for the telomere qPCR on a four-fold dilution series of sheep genomic DNA (10ng to 0.156 ng/reaction) at the optimal assay conditions.

Relative telomere length was calculated at two timepoints for blood samples collected from animals in the MAP and *H.contortus* co-challenge trial (3 weeks post-challenge (Measurement A) and 37 weeks post-challenge (Measurement B)) and at a single timepoint (7 days prior to challenge) for animals in the *Salmonella* trial. The change in relative telomere length between the two timepoints from the co-challenge trial was assessed (Fig. 4.3.16). There was a significant difference in relative telomere length measured in blood samples at 3 weeks post-challenge compared to 239 days later, at 37 weeks post-challenge; samples collected at the later timepoint had significantly shorter relative telomere length compared to the early timepoint (p=0.0066) A similar assessment was not done for the *Salmonella* trial as this was of short duration (10 days) and telomere length was not expected to change during this time period.
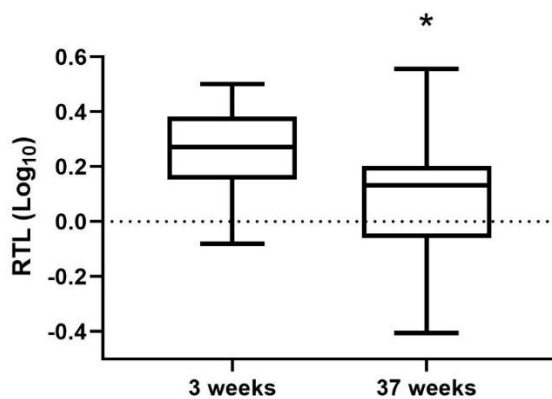
Figure 4.3.16. Box and whisker plot comparing the Log_{10} relative telomere lengths (RTL) at the two sampling time points 3 weeks (n=18) and 37 weeks (n=18), for sheep in the experimental co-challenge. * Significant difference (p=0.0066) between the relative telomere lengths at the timepoints.

In the commercial property (Arthursleigh) sheep trial, relative telomere length was assessed at two timepoints, on blood samples collected when the sheep were 4 months and 11 months of age. This samples set represents the stress associated with a normal, extensively grazed production system. Only a subset of the animals (n=44) that had the highest and lowest weight gain proportional to initial weight were analysed. Interestingly, relative telomere length was significantly longer at the later timepoint (Fig. 4.3.17). An increased telomer length in lambs over the first year of life has been previously reported (Shiels et al., 1999).
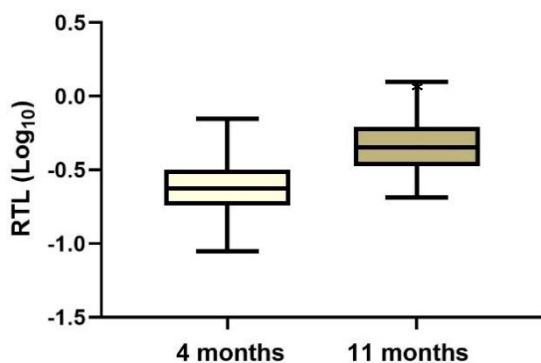
Figure 4.3.17. Box and whisker plot comparing the Log_{10} relative telomere lengths (RTL) at the two sampling time points 4 months (n=44) and 11 months (n=42), for sheep in the experimental co-challenge. * Significant difference (p<0.0001) between the relative telomere lengths at the timepoints.

The potential association between the relative telomere length and performance or disease variables in sheep was assessed. The variables include the ADG or proportional weight gain, the severity of disease for JD and barbers pole worm infections, and the *Salmonella* disease outcome

post-challenge. ADG results for sheep included in the co-challenge trial showed a positive correlation with RTL, with a longer telomere length at the earlier timepoint (3 weeks post-challenge) moderately correlated (r = 0.54 (95% confidence interval (CI) 0.10 to 0.80), p= 0.017) with improved ADG (Figure 4.3.18 A). No correlation was seen at the later timepoint in this trial. For the commercial property where there was no intentional disease challenge, no correlation was seen between the relative telomere length at 4 months (Fig. 3.3.18 B) or 11 months of age (data not shown) and weight gain proportional to the initial weight of the animals. The Spearman's correlation analysis also showed no significant correlation between relative telomere length and ADG at either 4 or 11 months of age in these sheep (data not shown).
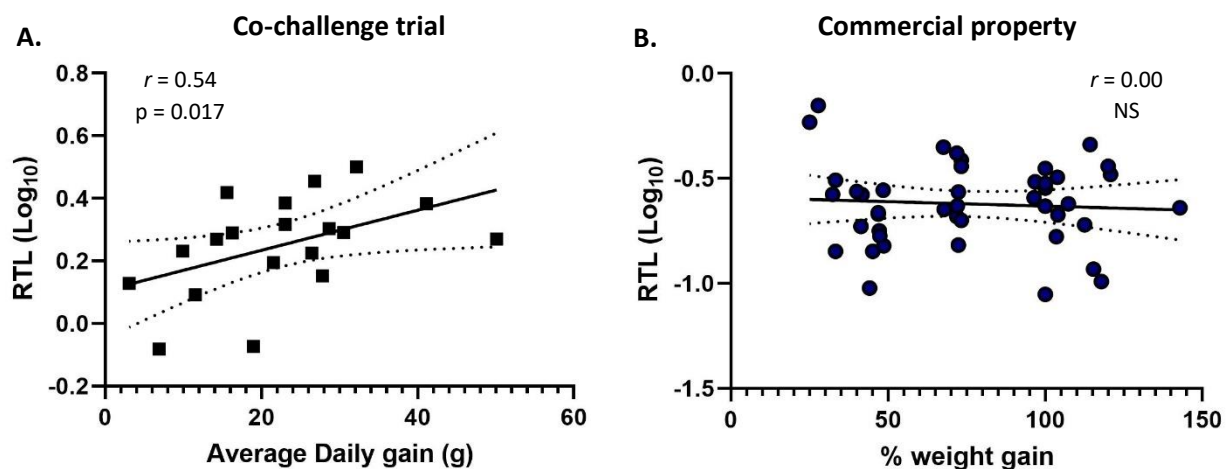
Figure 4.3.18. Scatter plots comparing the Log_{10} relative telomere lengths (RTL) and weight gain performance data. The Spearman's correlation analysis (r and p value) results are shown on each graph. Panel A shown the Log_{10} relative telomere lengths (RTL) versus Average Daily gain for sheep in the experimental co-challenge trial (n=18). The Spearman's correlation analysis (r and p value) results are shown. Panel B shows Log_{10} relative telomere lengths (RTL) versus the weight gain proportional to the initial weight (% weight gain) for animals from the commercial property (Arthursleigh) trial on sheep at 4 months of age. NS: Not significant.

When the relative telomere length for the sheep from the co-challenge and *Salmonella* challenge trials was compared to disease susceptibility, there was a consistent negative correlation between RTL length and severity of disease at the first timepoint assessed (Fig. 4.3.19). This was significant only for AFEC (r = -0.49 (95% CI -0.78 to -0.03, p = 0.034) (Figure 4.3.19 A), but was approaching significance for paratuberculosis score (r = -0.43 (95% CI -0.75 to -0.04), p = 0.064) (Figure 4.3.19 B). When the RTL results were sub-grouped into categories of disease severity from Resilient to Susceptible, there were no significant differences found between the groups, however there was a consistent trend towards lower average RTL measures in the Susceptible animals at the first timepoint measured (not shown).
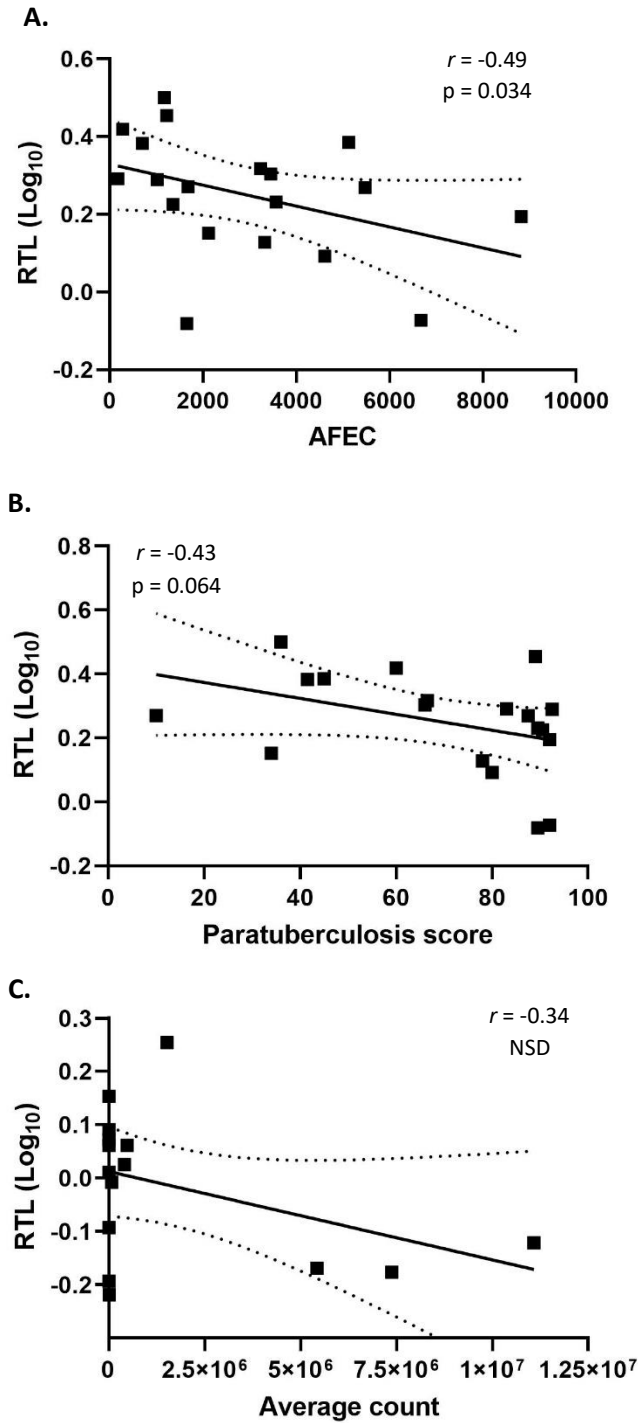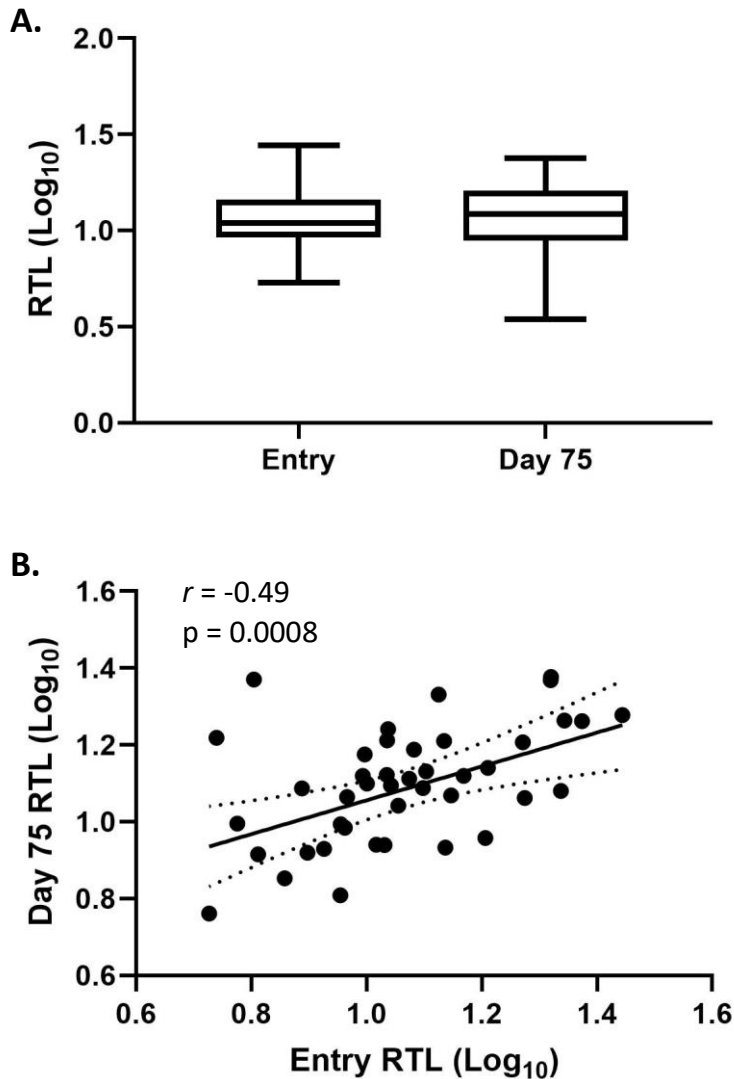
Figure 4.3.19. Scatter plots comparing the Log_{10} relative telomere lengths (RTL) and A. average faecal egg count for sheep from the co-challenge trial (n=18), B. Paratuberculosis score for sheep from the co-challenge trial (n=18), or C. Salmonella disease outcome (average colony counts) for sheep in the Salmonella challenge trial (n=16). The Spearman's correlation analysis (r and p value) results are shown on each graph.

Full results for this study are included in **Appendix 8.5**.

4.3.4.2 Relative telomere length and production outcomes in feedlot cattle

A similar study of relative telomere length was conducted in feedlot cattle, with testing conducted on blood samples collected at induction and at finishing 75 days later. There was no significant difference in RTL measured at the time of entry and relative telomere length measured at the final sampling timepoint prior to finishing, 75 days later, though the two measurements were moderately correlated, as shown in Fig. 4.3.20.

A.

B.

$r = -0.49$
$p = 0.0008$

Figure 4.3.20. Comparison of relative telomere length (RTL) measured on DNA from blood sampled at entry in into the feedlot and at finishing (Day 75). A. Box and whiskers plot of the Log_{10} RTL values at each timepoint. B. Scatterplot of Log_{10} RTL at entry and Day 75 showing correlation between the two values. The Spearman's correlation and *r* and p value are included on the graph and the regression line (solid) and 95% confidence interval (dotted line) are shown.

The breed and sex of the cattle did not show a significant effect in terms of RTL at entry to the feedlot (Fig. 4.3.21). Given this finding, data for all breeds and both sexes were analysed together.
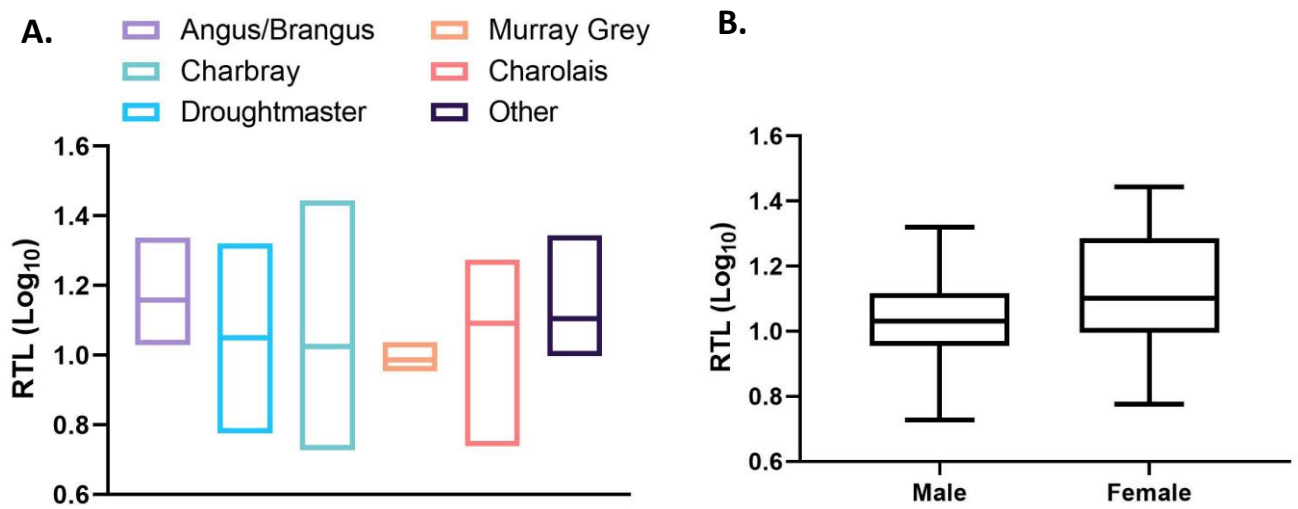
Figure 4.3.21. Comparison of RTL (Log_{10}) measured on blood DNA sampled at entry into the feedlot and A. breed or B. sex of the animals. No significant differences were found between the groups for each analysis.

A correlation analysis revealed a weak negative relationship between the weight gain proportional to entry weight (% weight gain) and RTL measured at the time of entry into the feedlot, as shown in Figure 6A. This inverse trend was consistent between the RTL measurement at entry (Figure 4.3.22 A) or at finishing (Fig. 4.3.22 B). There was no correlation between meat quality (MSA Index) and either RTL at entry (Fig. 4.3.22 C) or finishing (data not shown), though there was again an inverse slope for the regression line. There was no correlation between RTL at any timepoint and the entry weight of the animals (data not shown). The change in RTL measured at entry and finishing was also not correlated with any of the production measures.

Full results for this study are included in **Appendix 8.6**.
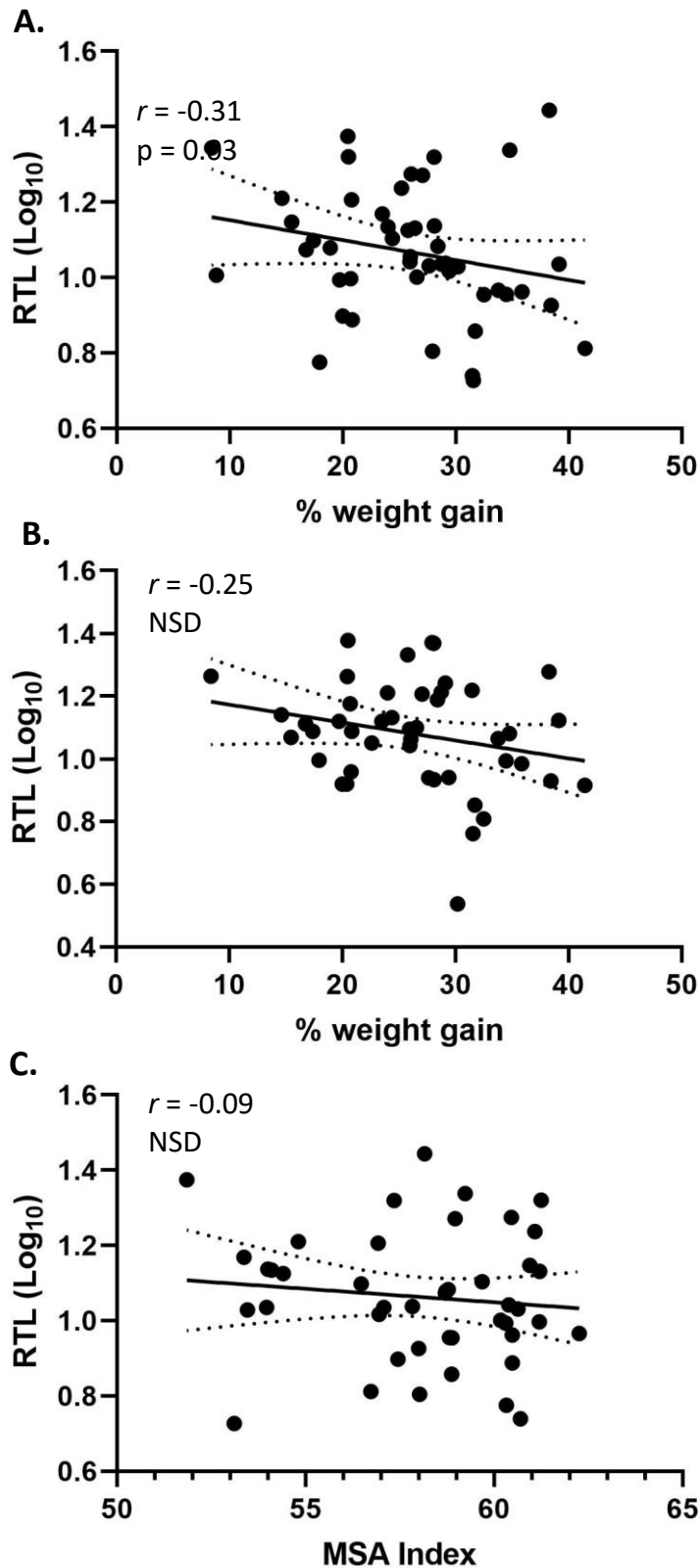
Figure 4.3.22. Relationship between relative telomere length (RTL) measured on DNA from blood sampled at entry in into the feedlot (A and C) or Day 75 (B) and production outcomes. A. and B. comparison was made to the weight gain proportional to entry weight (%weight gain), C. comparison to meat quality (MSA Index). For each comparison, a scatterplot of Log_{10} RTL at entry and the production measure is presented, showing the correlation between the two values. The Spearman's correlation and *r* and p value are included on the graph and the regression line (solid) and 95% confidence interval (dotted line) are shown. Only the RTL (Log_{10}) at entry and % weight gain had a significant, though low level, of correlation, however the inverse trend was a consistent finding. NSD: not significantly different.

5. Conclusion

5.1 Immune factors

5.1.1 Cytokines

Table 4.2.2 summarises significant differences in cytokine responses that differentiate between susceptibility to disease and weight gain. The cytokine responsiveness in sheep or cattle subjected to pathogen burden was overall greater in animals that were susceptible to disease; these cytokines included CXCL10, IFNγ, IL-1β and IL-8. When looking at weight gain, animals with a lower percentage weight gain were also more cytokine responsive; these cytokines included Decorin and TNFα. This overall pattern suggests that the ability to regulate pro-inflammatory cytokine responses are important to maintain health. This is further supported by the finding that cattle that gained more weight in the face of pathogen challenge had lower IFNγ responses than animals subjected to similar pathogen challenge and husbandry practices.

The protein array results suggested clear trends in protein expression, however ELISA tests were not available for some targets and the sensitivity for others was low, which meant that it was not possible to validate these putative biomarkers. For these reasons, we did not assess the findings in a larger cohort. At present, until technologies for livestock immunology improve, it is not cost-effective to pursue these biomarkers for inclusion in the immune fitness scorecard. Developments, for example in the field of Luminex bead technology and pen-side tests, may open opportunities in future for these biomarkers to be applied.

5.1.2 Cortisol

The results from this study demonstrated that there was a significant correlation between faecal and wool cortisol measurement in sheep and that physiological stress associated with haemonchosis is detectable through changes in the faecal cortisol levels. Though FCM and average FEC were moderately correlated, no correlation was seen with paratuberculosis disease outcomes in terms of faecal or wool cortisol levels. FCM is a reliable measure of the sheep stress response, however, it is important to include various factors such as biological variation, external stimuli and procedures to better validate FCM as a measure of sheep stress response.

In cattle, hair cortisol levels showed a high degree of variability between animals that needs to be further investigated. The animals that were burnt in the fires did seem to have elevated hair cortisol

levels. Cortisol measurements in hair of cattle have been previously reported as a marker of animal welfare (Vesel et al., 2020).

As the results for this marker showed that it was not strongly correlated with immune function, cortisol measures were not pursued as one of the biomarkers of interest in relation to immune fitness. However, as a means of assessing animal welfare and stress responses, both faecal and hair samples were shown to be appropriate to determine semi-acute or more chronic stress, respectively.

5.1.3 Trace minerals

The trace mineral detection in serum was conducted to determine if this may be an explanatory variable of immune fitness. However, there was no correlation found between disease severity measures and any of the trace minerals assessed. Interestingly, there was little correlation between trace mineral levels in the serum and production outcomes such as weight gain and meat quality in cattle. A number of the key minerals of interest were at baseline levels in the serum. In many cases blood is not the main storage site in the body for these minerals, indicating that tissue samples (such as kidney or liver) may be a more appropriate sample to test. However, these are not easily accessible ante-mortem samples and are usually measurements that are made post-mortem. As such, trace minerals were not incorporated as biomarkers of immune fitness.

5.2 Genomic correlates

5.2.1 Feedlot and Meta-analysis

The results of transcriptomic analysis of feedlot finished cattle and the subsequent meta-analysis of transcriptomic datasets primarily sourced from public data sources has facilitated identification of genes whose expression is correlated with immune fitness in terms of the production measures weight gain (ADG) or disease resilience in both sheep and cattle models (Objective 1a). Further, meta-analysis has enabled identification of genes at the arrival of cattle at the feedlot that was predictive of the capacity of achieving high meat quality as measured by the MSA index. These genes are suggested as putative markers of a baseline of red meat quality (Objective 3). Finally, deep-diving into the gene expression predicted functional profiles of cattle resulting in optimal production outcomes (high ADG or high MSA index score) and cross comparison with gene profiles associated with cattle experiencing exposure to stressors has facilitated insight to key pressure points (Objective 2)

There was clear differential gene expression between high ADG cattle and low ADG cattle, disease resilient cattle and sheep and those cattle with the capacity to achieve high meat quality as measured by the MSA index. The reproducibility and validity of a selection of these genes has been confirmed by qRT-PCR analysis on samples representative of standard Australian livestock production systems. Combinations of the above genes would be suitable inclusion into a panel (score card) to determine which animals have the best resilience to disease burden and/or higher immune fitness.

Differentially expressed genes were mapped to functional pathways involved in antigen presentation via MHCII CD4+ lymphocytes, Th1 and Th2 activation, carbohydrate and lipid metabolism. In brief, the immune status and metabolic pathways varied between high and low performing animals, and this variation was in part attributed to differentially expressed genes. These findings align with previously described gene functions associated with feed efficiency and residual feed intake of (Chen et al., 2011; Connor et al., 2010; Kern et al., 2016; Lindholm-Perry et al., 2020).

The principle component analysis of the array data in terms of ADG, MSA index score, breed type and farm of origin identifies a thought-provoking corollary; the variance in the samples suggests distinct separation between the high ADG animals from Farm 3 compared the others that are largely clustered with the low weight animals. This implies a farm of origin effect in relation to capacity to achieve high weight at the culmination of the 78-day finishing process. However, it is interesting to note that although the majority of the animals from Farm 3 transitioned well to the feedlot environment, the overall weight gain achieved by these steers of mixed breed was greater, and they transitioned to the feedlot conditions more efficiently than cattle from the other farms, this did not translate to meat consistently scored within the high range of the MSA index. This suggests management techniques on farm may play into genetic expression. Factors influencing genetic expression may include the on-farm ration, pathogen exposure, soil microbiome, climate, husbandry practices and duration of transport to feedlot. Further research analysing farm of origin factors is suggested. The individual animal microbiome should also be considered as a key factor affecting feedlot performance as the microbiome is responsible for the production of volatile fatty acids utilised for energy and weight gain (Dijkstra, Forbes & France, 2005). Variation within microbial populations will alter the metabolizable energy available or metabolic pathways activated due to substrate availability thus this is an area for future research. Further analysis of the production scores of all 80 cattle sourced from the four farms revel no significant association between ADG and meat quality (MSA index) however analysis of gene expression identified differentially expressed genes at induction that are predictive of cattle capable of achieving both high ADG and meat within

the top 25% of eating quality. Further analysis of the efficacy of these genes as potential candidates for markers of optimal production is warranted as it was not feasible within the scope of this project.

5.2.2 Telomere

The results show that relative telomer length represents a good candidate biomarker of animal welfare and immune fitness. A negative correlation between disease burden and relative telomere length was found that was significant for some outcome variables, suggesting that telomere length, as an objective measure of biological age and cumulative stress, may relate to measurable changes in the physiology of the sheep. The data supports the hypothesis that animals with shorter relative telomer lengths have increased susceptibility to disease. Decreases in relative telomere length when challenged with a chronic physiological stressor is a well-documented phenomenon in humans and animals (de Punder et al., 2019; Wilbourn et al., 2018; Yip et al., 2016). For sheep from a commercial property that was not subjected to an intentional disease challenge and were managed under normal extensive grazing conditions, there was no correlation between relative telomere length and weight gain. This suggests that selection of animals for disease resilience based on telomere length would not have a negative impact on performance in the production system in terms of weight gain.

In the feedlot setting, there was a weak inverse correlation between relative telomer length and weight gain in feedlot cattle over the finishing period, with a reduced telomere length related to higher weight gain. This was an unexpected result, as it was hypothesised that a longer telomere length may be associated with higher performance as these animals may be better able to cope with stress. The animals in this trial were exposed to unavoidable stress, due to transportation to the feedlot, environmental conditions within the feedlot, along with the stress related to the newly established hierarchical structure between cattle in the feedlot and access to food. A possible explanation for this unanticipated inverse relationship between telomere length and weight gain is that selective breeding programs have tended to focus on achieving high productive yields (Canario et al., 2013; Rauw & Gomez-Raya, 2015). Importantly, Herve et al. (Herve et al., 2020) found that if producers want to maximize production outcomes in beef cattle, they should consider disease factors and reduce stress by establishing protocols where groups from the same farm are maintained and minimizing transportation distance. The feedlot cohort investigated had low levels of disease presentation e.g. Bovine Respiratory Disease (BRD). It would be interesting to examine a larger cohort of animals and in particular cohorts where BRD is expressed, to determine any impact of a shorter telomere length on disease resilience and hence overall productive outcomes of these cattle. This may further elucidate the association between weight gain, disease susceptibility and relative telomere length in cattle. Examining if relative telomere length is correlated with other

outcomes, such as milk production and wool yield and characteristics, in a setting in which there was no intentional major physiological or environmental challenge is also important to further understand the relationship of telomere length with livestock production outcomes.

Our data suggests that the measurement of relative telomere length is correlated with disease outcomes and hence is a potential biomarker of immune fitness. Further, telomere length represents a biomarker that can potentially enable producers to determine the cumulative stress that husbandry procedures induce on livestock and how this affects their physiology.

5.3 Key findings

- Meta-analysis of seventeen transcriptomic datasets encapsulating the gene expression responses of sheep or cattle exposed to the pathogens *Brucella ovis* (sheep), *Mycobacterium bovis* (cattle), *Mycobacterium avium* subsp. *paratuberculosis* (sheep and cattle), and *Rhipicephalus microplus* (sheep and cattle), Pestivirus (cattle), *Neotyphodium coenophialum* (cattle), and acidosis revealed 165 genes meeting the criteria for differential expression associated with disease resilience in cattle across multiple breeds, sample sources and disease exposure states, similarly, 1437 genes are significantly differentially regulated in sheep achieving resilience to disease. Selections of these genes were validated by qRT-PCR.

- Combinations of the above genes would be suitable for inclusion into a panel (score card) to determine which animals have the best resilience to disease burden and/or higher immune fitness.

- Fifty-four genes meet the criteria for differential expression for cattle achieving both high ADG and a high MSA index score. These could be utilised as correlates of production outcome associated with immune fitness in feedlot cattle.

- Meta-analysis of public sourced transcriptomic databases combined with transcriptomic data sourced from cattle undergoing production stressors enabled in-depth comparison between the multiple environmental stressors with production outcomes (ADG, MSA index score and disease status) and identified adaptation to variation in nutrient source as a primary pressure point in determining the capacity of the animal to achieve optimal production outcomes.

- We have identified 226 genes meeting the criteria for differential expression either at induction of cattle to feedlot or consistently across both sampling times (Day 0 and day 76) that are associated to cattle achieving a high MSA index score in comparison to those who

achieved low score. The expression of these genes may serve as a baseline for optimal red meat quality metrics regardless of breed, farm of origin and exposure to environmental stress.

- Telomere length could be utilised as a biomarker to determine which animals have the best resilience to disease burden and higher immune fitness. Relative telomere length could additionally be used to determine best practice for livestock production.

- The protein array identified putative biomarkers for immune fitness, however, the technology is not currently available to facilitate cost-effective testing to adopt these. At this time, we do not recommend inclusion of these in the immune fitness scorecard.

- Cortisol (hair and faeces) and trace minerals were not strongly associated with immune or production outcomes and were not taken forward for this reason.

5.4 Benefits to industry

This program aimed to address key points in the production process which can impact on animal welfare. Animal welfare is a key concern to all stakeholders; farmers should aim to achieve high welfare standards to maintain high production rates, and the general public guides animal welfare standards by their purchasing power and opinion (Lusk & Norwood, 2008; Meunier et al., 2020). It is a priority area for the red-meat industry, representing an enormous risk to the industry, which is estimated to grow to more than $3 billion by 2030 (Meat Industry Strategic Plan 2020). Diseases of livestock have a significant impact on both animal welfare and livestock production, with hundreds of millions of dollars spent each year to prevent and treat disease, and millions in profit foregone due to production losses (GHD et al., 2015).

We have identified quantifiable measures of sustained health and welfare through delivery of a prototype scorecard for immune fitness. This provides an opportunity for the livestock industry to look holistically and address drivers that can affect productivity, regardless of production pressures that may be specific to a particular producer. The scorecard and associated findings can be applied in a number of ways:

- Enabling adaptation of handling and management strategies through application at pressure points, leading to improved animal welfare and economic benefits to the red meat industry.

- The **'Immune Fitness Index'** based on the panel of biomarkers included in the scorecard could serve as a marketable commodity at point of sale, with benefits to both the producer and the consumer.

- The biomarkers would facilitate identification of animals at risk of failure to thrive throughout the production chain and would be valuable to use from farm to post farm-gate.
- The **'Immune Fitness Index'** could be utilised to increase public confidence and improve perceptions around animal health and welfare in the livestock industries. This graded index could provide reassurance to consumers that animal welfare has not been compromised along the production chain.
- There were specific biomarkers associated with meat quality, many of which were aligned to immune fitness. These could be easily adopted by producers to facilitate selection of high performing livestock without compromising the health and welfare of the animal. This would have foreseeable financial benefits to the producer and would aid in adoption of the **Immune Fitness Index**.

6. Future research and recommendations

Working together with MLA, Industry and other livestock scientists, we propose further validation prior to adoption of the **Immune Fitness Index**. This should include validation of the relevance across breeds and production systems.

The gene signature(s) identified as predictors of Immune fitness and MSA Index could be incorporated into existing EBVs (Figure 6.1).

We have identified a putative farm-of-origin effect that is not explainable by breed or sex variations. This suggests environmental factors, possibly impacting internal biology, such as the microbiome (gut microbiota). As beef cattle move through a feedlot system their feed ration changes to a finishing diet with a higher proportion of grain compared to their pasture-based diet on farm. Consequently, the microbes within the rumen of these cattle must adapt to extract energy from the high concentrate feed as opposed to a high fibre ration. The relative proportions of microbe populations within the rumen are influenced by diet and have effects on productivity and systemic health of the cow. If the microbiome is poorly adapted to the diet, animal feed conversion will be decreased, and the animal will perform poorly. Digestive disorders such as acidosis can also occur elevating circulating proinflammatory cytokines increasing susceptibility to disease. With improvements in technology, it is now possible to better understand the modulation of gut microbiota in relation to feed conversion efficiency and the immune response. Characterising variations in the gut microbiome from farm of origin and throughout production systems may lead

to improvements in production performance and is fundamental to designing strategies to prevent disease and maintain healthy animals (see Fig. 6.1).
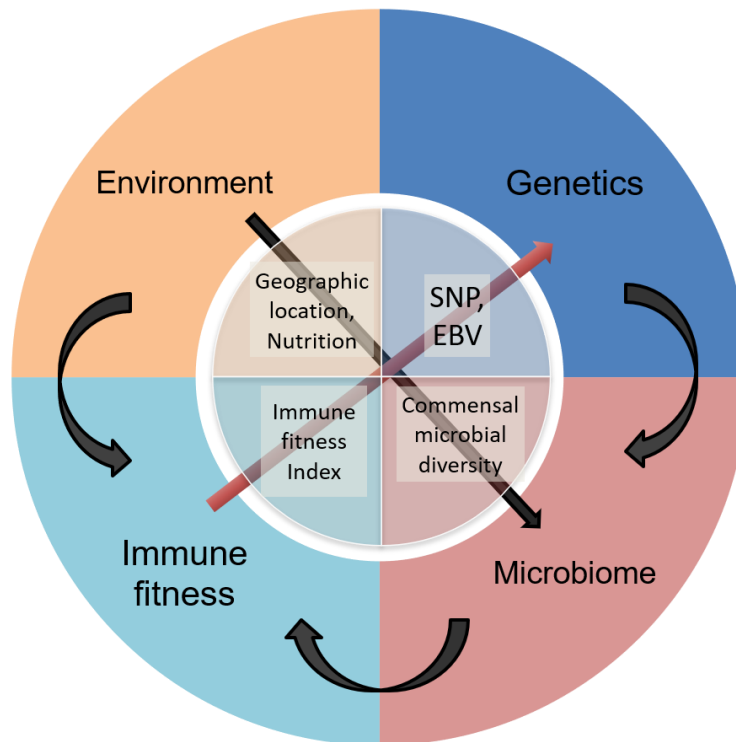

Figure 6.1. Incorporation of information from multiple aspects to achieve optimal animal welfare and production outcomes. There is an interplay between genetics, the microbiome and the environment with immune fitness. This interplay can be harnessed to the benefit of producers and to improve health and welfare of livestock. Immune fitness biomarkers can feed back to improve overall genetics through incorporation in EBVs (red arrow).

7. References

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8. Appendix

8.1 Identification of immune factors.

8.2 Long-term cortisol measurements to identify stress in sheep.

8.3 Transcriptomic analysis of feedlot finished cattle.

8.4 Genetic correlates of immunity: A meta-analysis.

8.5 Telomeres as a biomarker of immune fitness in sheep.

8.6 Telomere dynamics in feedlot cattle.