Acute phase proteins and biomarkers for health in chickens

Emily Louise O'Reilly

B.Sc. (Hon), BVMS, M.Sc.

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Ph.D.)

Institute of Biodiversity, Animal Health and Comparative Medicine
College of Medical, Veterinary and Life Sciences
University of Glasgow

February 2016

Abstract

Acute phase proteins (APPs) are proteins synthesised predominantly in the liver, whose plasma concentrations increase (positive APP) or decrease (negative APP) as a result of infection, inflammation, trauma and tissue injury. They also change as a result of the introduction of immunogens such as bacterial lipopolysaccharide (LPS), turpentine and vaccination. While publications on APPs in chickens are numerous, the limited availability of anti-sera and commercial ELISAs has resulted in a lot of information on only a few APPs.

Disease is a threat to the poultry industry, as pathogens have the potential to evolve, spread and cause rapid onset of disease that is detrimental to the welfare of birds. Low level, sub-acute disease with non-specific, often undiagnosed causes can greatly affect bird health and growth and impact greatly on productivity and profitability. Developing and validating methods to measure and characterise APPs in chickens will allow these proteins to be used diagnostically for monitoring flock health. Using immune parameters such as APPs that correlate with disease resistance or improvements in production and welfare will allow the use of APPs as selection parameters for breeding to be evaluated.

For APPs to be useful parameters on which to evaluate chicken health, information on normal APP concentrations is required. Ceruloplasmin (Cp) and PIT54 concentrations were found to be much lower in healthy birds form commercial production farms than the reported normal values obtained from the literature. These APPs were found to be significantly higher in culled birds from a commercial farm and Cp, PIT54 and ovotransferrin (Ovt) were significantly higher in birds classified as having obvious gait defects.

Using quantitative shotgun proteomics to identify the differentially abundant proteins between three pools: highly acute phase (HAP), acute phase (AP) and non-acute phase (NAP), generated data from which a selection of proteins, based on the fold difference between the three pools was made. These proteins were targeted on a individual samples alongside proteins known to be APPs in

chickens or other species: serum amyloid A (SAA), C-reactive protein (CRP), Ovt, apolipoprotein A-I (apo-AI), transthyretin (Ttn), haemopexin (Hpx) and PIT54.

Together with immunoassay data for SAA, Ovt, alpha-1-acid glycoprotein (AGP) and Cp the results of this research reveal that SAA is the only major APP in chickens. Ovotransferrin and AGP behave as moderate APPs while PIT54 and Cp are minor APPs. Haemopexin was not significantly different between the three acute phase groups. Apolipoprotein AI and Ttn were significantly lower in the HAP and AP groups and as such can be classed as negative APPs.

In an effort to identify CRP, multiple anti-sera cross reacting with CRP from other species were used and a phosphorylcholine column known to affinity purify CRP were used. Enriched fractions containing low molecular weight proteins, elutions from the affinity column together with HAP, AP and NAP pooled samples were applied to a Q-Exactive Hybrid Quadrupole–Orbitrap mass spectrometer (Thermo Scientific) for Shotgun analysis and CRP was not identified. It would appear that CRP is not present as a plasma protein constitutively or during an APR in chickens and as such is not an APP in this species.

Of the proteins targeted as possible novel biomarkers of the APR in chickens mannan binding lectin associated serine protease-2, α-2-HS-glycoprotein (fetuin) and major facilitator superfamily domain-containing protein 10 were reduced in abundance in the HAP group, behaving as negative biomarkers. Myeloid protein and putative ISG(12)2 were positively associated with the acute phase being significantly higher in the HAP and AP groups. The protein cathepsin D was significantly higher in both HAP and AP compared to the NAP indicating that of all the proteins targeted, this appears to have the most potential as a biomarker of the acute phase, as it was significantly increased in the AP as well as the HAP group.

To evaluate APPs and investigate biomarkers of intestinal health, a study using re-used poultry litter was undertaken. The introduction of litter at 12 days of age did not significantly increase any APPs measured using immunoassays and quantitative proteomics at 3, 6 and 10 days post introduction. While no APP was found to be significantly different between the challenged and control groups at anytime point, the APPs AGP, SAA and Hpx did increase over time in all birds. The protein apolipoprotein AIV (apo-AIV) was targeted as a possible APP and

because of its reported role in controlling satiety. An ELISA was developed, successfully validated and used to measure apo-AIV in this study. While no significant differences in apo-AIV plasma concentrations between challenged and control groups were identified apo-AIV plasma concentrations did change significantly between certain time points in challenged and control groups. Apoliporotein AIV does not appear to behave as an APP in chickens, as it was not significantly different between acute phase groups. The actin associated proteins villin and gelsolin were investigated as possible biomarkers of intestinal health. Villin was found not to be present in the plasma of chickens and as such not a biomarker target. Gelsolin was found not to be differentially expressed during the acute phase or as a result of intestinal challenge. Finally a proteomic approach was undertaken to investigate gastrocnemius tendon (GT) rupture in broiler chickens with a view of elucidating to and identify proteins associated with risk of rupture. A number of proteins were found to be differentially expressed between tendon pools and further work would enable further detailing of these findings.

In conclusion this work has made a number of novel findings and addressed a number of data poor areas. The area of chicken APPs research has stagnated over the last 15 years with publications becoming repetitive and reliant on a small number of immunoassays. This work has sought to characterise the classic APPs in chickens, and use a quantitative proteomic approach to measure and categorise them. This method was also used to take a fresh approach to biomarker identification for both the APR and intestinal health. The development and validation of assays for Ovt and apo-AIV and the shotgun data mean that these proteins can be further characterised in chickens with a view of applying their measurement to diagnostics and selective breeding programs.

Table of Contents

List of Tables

List of Figures

Acknowledgements

I would like thank my supervisor Professor David Eckersall for giving me the opportunity to return to university and undertake this PhD program. I have developed professionally and personally during this time and have really enjoyed doing this PhD, and while this is because of the interesting subject matter, it is also because of the relaxed and enthusiastic support from David, who has allowed me to approach this course of study in my own way. Undertaking a M.Sc. in the first half of this PhD and then having two babies a year apart in the final year added just a wee bit of pressure and has meant time lines for research and writing were at times fragmented! I would not have been able to do all these things and get this all together here without the support and guidance of such a fantastic supervisor – thank you.

In the lab my colleagues and friends Dr.'s Mark Braceland, Faizal Ghazali and Funmilola Thomas have been great companions on this journey. I started alongside Mark and welcomed Faizal and Funmi along the way, so it's a little strange to be the last one out of our group left to finish their PhD! Mary Waterston in the early years was also extremely helpful in settling me into the lab and Eilidh McCulloch, a great colleague in the lab is now a good friend. I would like to thank the other labs in which I worked during this PhD. Dr. Richard Burchmore and his colleagues at Glasgow Polyomics provided great advice and support for the proteomic work and technicians Suzanne Eadie and Alan Scott provided great technical support and advice. I was also welcomed by the Mass spectrometry Lab at the Universite de Liege where I undertook a COST action short term scientific mission with Dr. Gabriel Mazzucchelli. Technician Lisette Trzpiot was particularly welcoming and we obtained lots of data working through lots of samples. Making the second leg of this mission to Liege while heavily pregnant I was relieved to get home with just my data as oppose to a baby as well!

At the vet school I would like to thank Professor Maureen Bain for aiding me in sourcing samples and being super supportive and also Dr Tim Parkins and Dr. Claire Walls for advice and help with the statistics. At SRUC I would like to thank Professor Nick Sparks and Dr. Vicky Sandilands for providing samples and advice. I would also like to acknowledge Professor Narayan Rath of the Univeristy of Arkansas for providing me with the original ovotransferrin protocol. At Aviagen I would like to thank Dr. Richard Bailey who has provided a great deal of advice and help with samples and taught me a great deal about gut health and anatomy while sampling. I would also like to thank Dr. Kellie Watson my main Aviagen supervisor as well.

From years ago, though without whose faith in my learning the path to this point would without doubt have been either a lot harder or perhaps completely impassable I would like to acknowledge Mr Dawkins (sadly no longer with us) from my school days and Dr Clive Bullock and Dr. Kay Sharp from Roehampton University (formally University of Surrey). I got the opportunity to undertake a DEFRA Veterinary Training and Research Initiative scholarship while a vet student and I would also like to thank Professor Mike Stear for giving me this opportunity. In Mike's lab I got a great introduction to veterinary research and this stayed with me making the move from practice into research much easier a few years later.

My friends and family have been a constant source of support, encouragement and love. My extended family in Ireland the McGovern's: Mary, Terry, Aileen, David, Meadhbh, Rose and Art, thank you Xx. Mary has been a great support thinking nothing of flying over to help with the babies when disasters strike and Terry too has been great at fixing our house when it appears to be crumbling around us! My friends have all been great source of love and support over the years, thank you all so much! Particular mentions to Kate Harris, Kari Kissel and Dr's. Martha Kirby and Amanda Bradley-Stuart - love you guys, thank you!

My Mum, what to say.....! She is an inspiration and has given me so much unwavering support and love, telling me to just 'do what I want and enjoy'. I didn't take the normal well trodden path into university, leaving school working alongside A'levels and degrees till I got to the latter part of vet school was tough but did emphasises the luxury of time devoted only to study and lab work. I couldn't have done any of it without my Mum. Similarly she is a wonderful Grandmother and her help and support in looking after Shay and Gaby has enabled me finish this thesis on time.

My best friend and partner Guv has been great throughout, present at the conception of the PhD idea he is super supportive and an absolutely fantastic father and friend and his humour and optimism gets us both through the toughest of days (and nights). Shay and Gaby, arrived 13 months apart during the final stage of the PhD, which (as well as making me wonder what the hell we did with our time before they came along) did make the last part of the PhD rather tough, it's been such a great journey but this final part could not have happened without Guv's love and support and I am grateful. Lastly Shay and Gaby thank you for doing what you do best – giving us lots of love and happiness and keeping us busy.....I hope I deleted all the "asaadccczzzz\\zzz" that Shay helpfully added when the laptop was unattended!!

Love and thanks to you all.

Author's Declaration

This thesis and work contained within it, was conducted from October 2010 to February 2016 under the supervision of Professor P. David Eckersall, of the University of Glasgow. The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged

Emily O'Reilly

February 2016

Dedications

Dedicated to my Mum, Guv, precious children Shay and Gaby and of course.....

..... the Songbird!

Songbird

Talking to the songbird yesterday
Flew me to a place not far away
She's a little pilot in my mind
Singing songs of love to pass the time

Gonna write a song so she can see
Give her all the love she gives to me
Talk of better days that have yet to come
Never felt this love from anyone

She's not anyone

A man can never dream these kinds of things
Especially when she came and spread her wings
Whispered in my ear the things I'd like
Then she flew away into the night

Gonna write a song so she can see
Give her all the love she gives to me
Talk of better days that have yet to come
Never felt this love from anyone

She's not anyone

Oasis

List of Publications and Presentations

Some of the work contained in this thesis has been the subject of, or is related to, the following publications or presentations:

Papers

O'Reilly, E. L. & Eckersall, P. D. (2014) *Acute phase proteins: a review of their function, behaviour and measurement in chickens* World's Poultry Science Journal **70** (1) 27 – 44

Ovotransferrin radial immuno-diffusion assay (Chapter 3)

Najafi, P., Zulkifli, I., Soleimani, A. F & Kashiani, P (2015) *The effect of different degrees of feed restriction on heat shock protein 70, acute phase proteins,and other blood parameters in female broiler breeders* Poultry Science **94**:2322-2329

Najafi, P., Zulkifli, I., Nurfarahin, A. J., Soleimani, A. F., Aryani, A. A., O'Reilly, E. L. & Eckersall, P.D. (2015) *Environmental temperature and stocking density effects on acute phase proteins, heat shock protein 70, circulating corticosterone and performance in broiler chickens* International Journal of Biometeorology **59**:1577-1583

Zulkifli, I., Najafi, P., Nurfarahin, A. J., Soleimani, A. F., Kumari, S., Aryani, A. A., O'Reilly, E. L. & Eckersall, P.D. (2014) *Acute phase proteins, interleukin 6 and heat shock protein 70 in broiler chickens administered with corticosterone*. Poultry Science **93**: 3112-318

Shakeri, M., Zulkifli, I., Soleimani, A. F., O'Reilly, E. L., Eckersall, P. D., Aryani, A. A., Kumari, S. & Abdullah, F. F. J. (2014) *Response to dietary supplementation of L-glutamine and L-glutamate in broiler chickens reared at different stocking densities under hot, humid tropical conditions*. Poultry Science **93**: 2700-2708

Thesis

O'Reilly, E.L. (2013) *The effect of microbial challenge on the intestinal proteome of broiler* chickens M.Sc. Scotland's Rural College

Conference proceedings

2015 69th Annual Conference of the Association for Veterinary Teachers and Research Work, (AVTRW)

The measurement of chicken acute phase proteins using a quantitative proteomic approach, *Chester, UK*

2014 4th Meeting of the COST Action on Farm Animal Proteomics

The measurement of chicken acute phase proteins Serum amyloid-A, C-reactive protein, Ovotransferrin, Tranthyretin, Haemopexin and Apolipoprotein A-1 using a quantitative proteomic approach, *Milan, Italy*

2013 3rd Meeting of the COST Action on Farm Animal Proteomics

The effect of microbial challenge on the intestinal proteome of broiler chickens, *Kosice, Slovakia*

2013 World Poultry Science Association (WPSA) UK branch annual meeting
The effect of microbial challenge on the intestinal proteome of broiler chickens, *Nottingham, UK*
O'Reilly, *et al.* (2013) *British Poultry Abstracts* **9**: 5 – 6

2012 XXIV World's Poultry Congress
The effects of meloxicam on the serum acute phase proteins ceruloplasmin, PIT54 and ovotransferrin in gait score 1 and gait score 3 broiler chickens, *Salvador, Brazil*

2012 World Poultry Science Association (WPSA) UK branch annual meeting
The effects of meloxicam on the serum acute phase proteins ceruloplasmin, PIT54 and ovotransferrin in gait score 1 and gait score 3 broiler chickens, *Nottingham, UK*
O'Reilly *et al.* (2012) *British Poultry Abstracts* **8**: 6-7

2012 2nd Meeting of the COST Action on Farm Animal Proteomics.
The serum proteome and acute phase proteins of broiler chickens with gait abnormalities, *Vilamoura, Portugal*

2011 Universitas 21 Graduate Conference on Food.
Acute phase proteins in broiler chickens, *Kuala Lumpar, Malaysia*

Awards & travel grants

COST Farm animal proteomics: Short term Scientific Mission (2013/2014) *Liege, Belgium*

WPSA President's prize for best presentation (2013)

COST Farm Animal Proteomics Runner up - Best poster category (2013)

British Veterinary Poultry Association travel bursary (2012)

WPSA summer scholarship (2012)

Houghton Trust travel grant (2012)

Funding

BBSRC CASE Studentship - BB/H016171/1

Aviagen Ltd

List of Abbreviations

%	Percent
+	Plus
+ve	Positive
-	Minus
-ve	Negative
±	Plus minus
<	Less than
>	More than
AGP	Alpha 1 acid glycoprotein
µg	Microgram
µg/ml	Microgram per litre
µl	Microlitre
° C	Degrees Celsius
1DE	One-dimensional
2DE	Two dimensional
ACN	Acetonitrile
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
AP	Acute phase
Apo-AI	Apolipoprotein A-1
Apo-AIV	Apolipoprotein AIV
APP	Acute phase protein
APR	Acute phase response
APRP	Acute phase reactive plasma
AIR	Acquired immune system
AU	Arbitrary units
BSA	Bovine serum albumin
Ca2+	Calcium ion
CHAPS	3[(cholamidopropyl)dimethylammonio]-1-propane sulphonate
Cl-	Chloride ion
CRP	C-reactive protein
Cp	Ceruloplasmin
Cu	Copper
Cy	Cyanine dye
Cx	Control
DDA	Data dependant acquisition
dH20	Distilled water
DIA	Data independant acquisition
DiGE	Difference gel elctrophoresis
DTT	Dithiotriethol
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionisation

ESI-MS	Electrospray ionization mass spectrometry
Fb	Fibrinogen
Fn	Fibronectin
FPLC	Fast protein liquid chromatography
GS	Gait score
g	Gram
g/L	Gram per liter
HAP	Highly acute phase
Hb	Haemoglobin
H2O2	Hydrogen peroxide
Hb	Haemoglobin
HCl	Hydrochloric acid
HDL	High density lipoprotein
Hp	Haptoglobin
Hpx	Haemopexin
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IB	Infectious bronchitis
IBDV	Infectious bursal disease virus
ID	Identification
IEF	Immunoelectrophoresis
Ig	Immunoglobulin
IIR	Innate immune response
IL	Interleukin
ILTV	Infectious laryngotracheitis virus
IPG	Immobilized pH gradient
IT	Incidence of rupture
kDa	Kilo Dalton
L	Litre
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LPAI	Low pathogenic avian influenza
LPS	Lipopolysaccharride
M	Molar
M2	Meloxicam 2mg/kg
M5	Meloxicam 5mg/kg
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MDL	Minimum detection limit
MHC	Major histocompatibility complex
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MALDI-	Matrix-assisted laser desorption ionization-time of flight tandem

TOF MS/MS	mass spectrometry
Mw	Molecular weight
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
n	Sample size
Na2+	Sodium ion
Na2CO3	Sodium carbonate
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NAP	Non-acute phase
NaOH	Sodium hydroxide
NCP	Nitroceullulose paper
No.	Number
NSAIDs	Non-streroidal anti-inflammatory drugs
OD	Optical density
Ovt	Ovotransferrin
PAGE	Polyacrylamide gel electrophoresis
pH	Power of hydrogen
pI	Isoelectric point
PMN	Polymorphonuclear
PO42-	Phosphate ion
PTM	Post-translational modification
p-value	Probability that null hypothesis is true
QC	Quality control
RID	Radial immunodiffusion
RBC	Red blood cell
RT	Ruptured tendon
S	Saline
SAA	Serum amyloid A
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sp.	Species
SRUC	Scotland's Rural College
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TIBC	Total iron binding capacity
TNF	Tumour necrosis factor
Ttn	Transthyretin
SRID	Single radial immunodiffusion
TBG	Thyroxine binding globulin
TBS	Tris buffered saline
TH	Thyrid hormone
TOF	Time-of-flight
UK	United Kingdom

UV	Ultraviolet
V	Volt
vs.	Versus
v/v	By volume
w/v	By weight

Chapter 1

General Introduction

1.1 The innate immune system

Multi-cellular organisms appeared about 525 million years ago and over time developed many specialized structures and biochemical pathways that constitute an innate immune response (IIR), which protected against pathogens before the adaptive immune response (AIR) appeared in jawed vertebrates about 75 million years later (Stavitsky, 2007). As a result, many aspects of the IIR are phylogenetically conserved among higher vertebrates with many genes and proteins of ancient vintage, conserved from invertebrate to vertebrate phyla (Stavitsky, 2007). As the first line of defence, the IIR is dedicated to containment, holding infections to a level that can be resolved by the ensuing development of the AIR. The IIR is non-specific and rapid, transpiring almost immediately after recognition of an invading pathogen. Consisting of both constitutive and inducible components the IIR is highly diverse and can directly control the replication or spread of bacteria and viruses through induction of phagocytosis or antimicrobial products (Kogut, 2009). The constitutive components include physical barriers (e.g. epithelial lining and mucus), neutro/heterophils, macrophages and other phagocytes, various antimicrobial peptides and proteins, complement and toll-like receptors which serve to prevent infection, eliminate potential pathogens, and initiate the inflammatory response (Klasing, 2004; Cray *et al.*,2009)

The AIR in contrast is target specific but it takes longer to develop. Adaptive immunity mostly operates through communication between antigen presenting cells, B-cells and T-cells by direct cell to cell contact using MHC, T-cell receptors and immunoglobulins and/or cytokines (Zekarias *et al.*, 2002; Kogut, 2009). Innate immunity provides instruction for the AIR through the activation of intracellular signalling pathways that initiate cellular processes such as the production of pro-inflammatory and/or anti-inflammatory cytokines/chemokines and the production of co-stimulatory molecules required for antigen presentation to the acquired immune system (Swaggerty *et al.*, 2006). Production of pro-inflammatory cytokines is stimulated by macrophages, following activation by bacterial products, they have a key role in initiating an IIR and assist in generating a local inflammatory response and the inducement of the acute phase response (APR) (Giansanti *et al.*, 2006; Swaggerty *et al.*, 2008)

1.1.1 The acute phase response

The inducible component of the IIR is the systemic APR (Klasing, 2004). The APR is observed across all animal species. It is a core part of the IIR with counterparts of the mammalian APR identified in invertebrates and fish, in which the APR is proposed to be more robust than that in mammals to compensate for a less-evolved adaptive immune response (Cray *et al.*, 2009). The APR is the prominent and non-specific systemic reaction of the innate immune system to local or systemic disturbances caused by trauma, infection, stress, surgery, neoplasia or inflammation the goal of which is re-establishment of homeostasis and healing (Gruys *et al.*, 2005; Cray *et al.*, 2009; Eckersall and Bell, 2010).

At the site of infection or place of tissue injury pro-inflammatory cytokines and chemokines are released by a variety of cell types, but mainly by macrophages (Murata *et al.*, 2004; Gruys *et al.*, 2005). Cytokines and chemokines are protein and peptide mediators that play a key role in immune and inflammatory responses through activation and regulation of other cells and tissues (Wigley and Kaiser, 2003). The cytokines that play a key role in the induction of the APR in both mammals and avian species are the pro-inflammatory cytokines. In chickens the pro-inflammatory cytokines are IL-1ß, IL-6, IL-17 family and TNF-α (Giansanti *et al.*, 2006). Cytokines and chemokines initiate and modulate the APR, and together with nitric oxide and glucocorticoids trigger the hepatic APR. Within the first few hours of an APR, protein synthesis within the liver and hepatocyte secretion is drastically altered and there are measureable changes in the plasma concentration of several plasma proteins referred to as acute phase proteins (APPs) (Gruys *et al.*, 2005). Diffusing into the extracellular fluid and circulating in blood, cytokines also activate receptors on different target cells leading to a systemic APR and resulting activation of the hypothalamic–pituitary–adrenal axis, reduction in growth hormone secretion and a number of physical changes clinically characterised by pyrexia, anorexia and catabolism of muscle cells. Other changes include decreased low and high density lipoproteins, leukopenia, increased ACTH, increased glucocorticoids, activation of complement and blood coagulation, decreased calcium, zinc, vitamin A and α-tocopherol (Gruys *et al.* 2005). Bacterial infections usually lead to a strong systemic APR due to the reaction of the mononuclear-phagocytic system's cells.

TNF-α and IL-1ß are induced in response to endotoxin. Thus endotoxins such as Lipopolysaccaride (LPS) from the cell wall of Gram negative bacteria are used to induce experimental APRs. Viral infections generally cause a milder APR (Gruys *et al*. 2005).

1.1.2 Acute phase proteins

The cytokines TNF-α, IL-1ß and IL-6 are the major mediators of the APR with IL-6 the major mediator for the hepatocyte secretion of most of the APP (Murata *et al*. 2004; Gruys *et al*. 2005; Cray *et al*. 2009). Hepatic mRNA of the APPs that increase during an APR is upregulated and there is a reduction of mRNA synthesis of those APPs that decrease during an APR (Gruys *et al*. 2005). These are termed positive APPs or negative APPs respectively. Albumin is the most abundant constitutive plasma protein (Murata *et al*., 2004) and represents a major negative acute APP. Due to selective loss by the renal and gastrointestinal systems or decreased hepatic synthesis, plasma albumin concentration decreases during an APR. In chickens, albumin concentrations decrease to 50 - 75 % of normal concentrations (Grieninger *et al.,* 1986; Adler *et al.,* 2001). The decrease in albumin synthesis is postulated to allow for the unused pool of amino acids to instead be used to generate positive APP and other important mediators of inflammation (Cray *et al*., 2009).

Positive APPs are further classified as minor, moderate or major APPs according to the magnitude and duration of increase during the APR. Major APPs increase 10 – 1000 fold, moderate APPs increase 4 - 10 fold and minor APPs represent those with only slight 2 – 3 fold increases. There are noted differences in the timing and duration of increase in positive APP. Major APPs tend to increase markedly within the first 48 hours after of the triggering event and decline rapidly due to a short half life. Moderate and minor proteins tend to increase more slowly and have more prolonged duration (Cray *et al*, 2009 Eckersall and Bell, 2010).

APPs are involved in the restoration of homeostasis and as such have varied functions that include opsonisation, activating complement, binding cellular remnants and neutralising enzymes, scavenging free haemoglobin, iron and free radicals, antibacterial, antiviral and anti oxidant and are modulators of the host's immune response. Acute phase proteins are involved in many crucial

metabolic and immune pathways and have roles that include scavenging extracellular haemoglobin, iron and free radicals and direct antibacterial and antiviral activity. The circulating concentrations of the APP are usually related to the severity of the disorder and the extent of the tissue damage. Quantification of their concentration can therefore provide diagnostic and prognostic information and assessment of the response to the triggering event (Murata *et al.*, 2004; Gruys *et al.*, 2005; Cray *et al.*, 2009). Acute phase proteins are recognized for their application to human diagnostic medicine having value in the diagnosis and prognosis of cardiovascular disease, autoimmunity, organ transplant and cancer treatment (Cray *et al.*, 2009), as well as providing general biomarker of health.

The serum concentrations of pro-inflammatory cytokines increase within a few hours of an initiating stimulus and are usually cleared from the circulation within a few hours. The APP serum concentrations are detectable for longer periods, generally the maximum response is seen at 24 – 48 hours after the initiation and the decline coincides with recovery from the infection. As alterations in plasma APP concentrations tend to remain longer they have a better diagnostic potential than cytokines (Petersen, *et al.* 2004). Acute phase protein profiles of both companion and farm animal species are well documented, yet it was the early 1990s before the wider application APP in veterinary medicine was reported. Since then, APPs have been used in a variety of veterinary species for monitoring herd health and companion animal diagnostics, where these clinical parameters are used diagnostically and for understanding the pathogenesis of important diseases (Petersen *et al.*, 2004; Cray *et al.*, 2009; Eckersall and Bell, 2010). Acute phase proteins, the degree to which they change during an APR, the cytokine profiles and the way these inflammatory mediators respond to various pathogenic challenges vary between species.

1.1.3 Acute phase proteins in chickens

The APR and the APP changes that occur in chickens in response to disease have been documented in a large number of studies as reviewed by O'Reilly & Eckersall (2014). Table 1.1 details the chicken APPs documented to date, their reported basal concentrations and using the mammalian classification as described in Eckersall and Bell (2010) the reported change during an APR. This

table briefly lists the functions of these APPs together with the page number where these APPs are introduced in further detail in the relevant chapters. Similarly other APPs, not yet characterised in chickens are also detailed here. Chickens represent an important avian species owing to their importance as a food production animal and studies have sought to identify and quantify APPs in chickens with the aim of establishing how these APPs change during different disease processes. Table 1.2 details viral, bacteria, parasitic, immune mediated diseases in which APP have been studies in chickens. Studies that have studied APPs in relation to welfare and behaviour are also included.

APP	Functions	References	Normal serum concentrations	Reference	Classification	Page
Albumin Al	• Regulate plasma osmotic pressure • Bind /transport endo/exogenous compounds • Antioxidant	Sitar *et al*. 2013	11.08 ± 1.34 g/L 20 g/L (Plasma) 15 g/L (Serum)	Inoue *et al.*, 1997 Hrubec and Whichard, 2002 Hrubec and Whichard, 2002	Negative	-
α1-acid Glycoprotein AGP	• Anti-inflammatory • Inhibit heterophil activation • Clear LPS and neutralise toxicity • Immunomodulatory • Plasma transport	Murata, *et al*. 2004; Ceciliani & Pocacqua, 2007	161.8 ± 25.8 mg/L 228 ± 20 mg/L	Inoue *et al.*, 1997 Takahashi *et al.*, 1998	Moderate	131
Ceruloplasmin Cp	• Copper storage and transport • Antioxidant - prevents iron induced oxidative stress	Floris *et al.*, 2000	30 mg/L	Disilvestro and Harris, 1985	Minor / Moderate	59
Fibrinogen Fb	• Coagulation • Pro-inflammatory	Davalos & Akassoglou 2012	0.99 (± 0.11) g/L 3.379 (± 0.485) g/L 2.8 (± 0.05) g/L	Georgieva *et al.*, 2010 Nazifi *et al.*, 2010 Amrani *et al.*, 1986	Minor	-
Fibronectin Fn	• Cell adhesion, migration, growth and differentiation in the extracellular matrix • Wound healing	Labat-Robert, 2012	0.31 (± 0.4) g/L	Amrani *et al.*, 1986	Minor	-
Haemopexin Hpx	• Sequester and transport haem • Iron homeostasis • Antioxidant	Ascenzi *et al.*, 2005; Tolosano *et al.*, 2010	0.15 g/L	Buyse *et al.*, 2007	Minor	139
Mannan binding lectin MBL	• Bind microorganisms • Phagocytosis • Immunomodulatory • Immunoglobulin secretion. • Activate complement system through distinct MBL pathway	Nielsen *et al.*, 1999; Juul-Madsen *et al.*, 2003; Schou *et al.*, 2010	0.4 – 37.8 mg/L (mean 5.8 (± 4.0) mg/L) Over 10 weeks old	Laursen and Nielsen, 2000	Minor	186*
Ovotransferrin Ovt	• Sequester, store and transport iron • Antibacterial • Immunomodulatory	Giansanti *et al.* 2007; Xie, *et al.* 2002b;	0.90 ±0.06 g/L 1.18 (± 0.13) g/L	Xie *et al.*, 2002b Rath *et al.*, 2009	Moderate	64

PIT54	• Bind haemoglobin • Anti-bacterial • Anti-inflammatory • Potent antioxidant	Iwasaki *et al.* 2001; Ceron *et al.*, 2005	0.100 (± 0.020) g/L 1.2 g/L 0.09 g/L 0.05 g/L	Georgieva *et al.*, 2010 Millet *et al.*, 2007 Nazifi *et al.*, 2011 Nazifi *et al.*, 2010	Minor	61
Serum amyloid A SAA	• Lipoprotein transport and metabolism • Prevent oxidative tissue damage • Recruit immune cells to areas of inflammation • Immunomodulatory	Eriksen *et al.*, 1993; Landman, 1998; Uhlar & Whitehead, 1999)	0.15 ± 0.02 mg/L 0.166 ±0.17 mg/L 1.590 ± 0.0041 mg/L 1.56 ± 0.13 mg/L	Alasonyalilar *et al.*, 2006 Sevimli *et al.*, 2005 Nazifi *et al.*, 2010 Nazifi *et al.*, 2011	Major	127
APPs uncharacterised in chickens						
C-reactive protein CRP	• Recognise and eliminate pathogens • Clear cellular and nuclear debris • Bind apoptotic cells • Amplify the classical pathway of complement activation	Volanakis, 2001;Kravitz *et al.*, 2005			Major (Humans, pigs, dogs) Minor (ruminants)	129
Apolipoprotein AI Apo-AI	• Antioxidant • Prevent lipid peroxidation • Cardioprotective • Redistribution of cholesterol	Franceschini 2003; Kravitz *et al.* 2005; Roman *et al.*, 2009			Negative	135
Apolipoprotein AIV Apo-AIV	• Lipoprotein metabolism • Control satiety • Anti-oxidant • Anti-inflammatory	Vowinkel *et al.*, 2004; Tso & Liu 2004			Positive (mice)	195
Transthyretin Ttn	• Bind thyroid hormones	Yamauchi & Ishihara, 2009			Negative	137
Fetuin	• Regulates calcium metabolism • Anti-inflammatory	Olivier *et al.*, 2000; Gangneux *et al.*, 2003; Dabrowska *et al.*, 2015			Negative Positive	186*

Table 1.1: Basal levels of chicken APPs and their described behaviour in chickens together with functions of other APPs, not yet characterised in chickens. The thesis page number listed indicates where this APP is introduced fully or discussed*.

	Infectious agent	APP	Reference
Viral	Infectious bursal disease virus	Cp	Nazifi *et al.*, 2010
		SAA	Nazifi *et al.*, 2010
		PIT54	Nazifi *et al.*, 2010
		Fb	Nazifi *et al.*, 2010
		Ovt	Xie et al., 2002b
		MBL	Nielsen, *et al.*, 1998a; 1999
	Infectious bronchitis virus	SAA	Nazifi *et al.*, 2011
			Asasi et al., 2013
			Seifi *et al.*, 2014
		Ovt	Xie *et al.*, 2002b
		AGP	Nakamura *et al.*, 1996
		MBL	Juul-Madsen *et al.*, 2003; 2007
		PIT54	Seifi *et al.*, 2014
			Asasi et al., 2013
		CRP	Seifi *et al.*, 2014
	Respiratory enteric orphan virus	Ovt	Xie *et al.*, 2002b
	Fowl Poxvirus	Ovt	Xie *et al.*, 2002b
	Infectious laryngotracheitis virus	Ovt	Xie *et al.*, 2002b
		AGP	Nakamura *et al.*, 1996
		MBL	Nielsen, *et al.*, 1998b; 1999
	Low pathogenic avian influenza	AGP	Sylte & Suarez, 2012
		Ovt	Sylte & Suarez, 2012
	Newcastle disease virus	SAA	Firouzi, *et al.*, 2014
		PIT54	Firouzi, *et al.*, 2014
Bacterial	*Escherichia coli* (LPS)	Cp	Butler *et al.*, 1972
			Takahashi *et al.*, 2009
		Ovt	Xie *et al.*, 2002b
			Hallquist and Klasing, 1994
		AGP	Takahashi *et al.*, 1994; 1995; 2009
			Nakamura *et al.*, 1998
			Buyse *et al.*, 2007
		Hpx	Barnes *et al.*, 2002
			Buyse *et al.*, 2007
		CRP	Patterson and Mora 1964; 1965
	Salmonella ser Typhimurium (LPS)	Cp	Song *et al.*, 2009
			Koh *et al.*, 1996
		Ovt	Xie *et al.*, 2000
		Hpx	Adler *et al.*, 2001
		PIT54	Millet *et al.*, 2007
		MBL	Millet *et al.*, 2007
	Salmonella ser Typhimurium	PIT54	Yazdani *et al.*, 2015
		SAA	Yazdani *et al.*, 2015
	Salmonella ser Gallinarum	Cp	Garcia *et al.*, 2009
		PIT54	Garcia *et al.*, 2009
		Ovt	Garcia *et al.*, 2009
		Hpv	Garcia *et al.*, 2009
	Staphylococcus aureus	SAA	Chamanza *et al.*, 1999
			Upragarin, 2005
		CRP	Patterson and Mora, 1964
	Pasteurella multocida	MBL	Schou *et al.*, 2010
		CRP	Patterson and Mora, 1964
	Gallibacterium anatis	Ovt	Roy *et al.*, 2014

	Streptococcus equi subsp. *zooepidemicus*	Ovt	Roy *et al*., 2014
	Mycoplasma gallisepticum	AGP	Peebles *et al*., 2014
Parasitic	*Eimeria tenella*	Cp	Georgieva *et al*., 2010
		PIT54	Georgieva *et al*., 2010
		Fb	Georgieva *et al*., 2010
		Ovt	Rath *et al*., 2009
		Cp	Richards and Augustine, 1988
	Eimeria maxima	Ovt	Rath *et al*., 2009
	Eimeria acervulina	Cp	Richards and Augustine, 1988
	Histomoniasis	CRP	Patterson Mora, 1964
	Ascaridia galli	MBL	Dalgaard *et al*., 2015
		CRP	Dalgaard *et al*., 2015
Immune mediated	Autoimmune vitiligo	Ovt	Rath *et al*., 2009
Metabolic	Tibial dyschondroplasia	Ovt	Rath *et al*., 2009
	Pulmonary hypertension	Ovt	Rath *et al*., 2009
	Femoral head separation	Ovt	Durairaj *et al*., 2009
Welfare & Behaviour	Egg laying system	AGP	Salamano *et al*., 2010
		Al	Salamano *et al*., 2010
	Broiler production system	AGP	Tuyttens *et al*., 2008
	Stocking density	Ovt	Shakeri *et al*., 2014; Najafi *et al*., 2015
		Cp	Shakeri *et al*., 2014; Najafi *et al*., 2015
		PIT54	Shakeri *et al*., 2014; Najafi *et al*., 2015
	Feed restriction	Ovt	Najafi *et al*., 2015
		Cp	Najafi *et al*., 2015
		PIT54	Najafi *et al*., 2015

Table 1.2: Studies of bacterial, viral, parasitic, immune-mediated and metabolic diseases and welfare in chickens that have measured APPs (adapted from O'Reilly & Eckersall, 2014).

Research into animal APPs has followed a general trend of identifying known human APPs in animal sera, establishing validated methods of measurement and then determining the response these APPs have to an infection in that animal species. This approach has also been applied to chickens with all of the recognised APPs in chickens having been previously identified in humans and other animals. Many of the studies in APP in chickens in Table 1.1 and 1.2 utilise LPS to stimulate an APR, from which the changes in an APP concentration are then measured. Other studies describe the development of methods to measure an APP in chickens or cite papers that have. Some studies utilise commercially available immunoassay kits with stated cross reactivity to chickens. However there are published studies that have undertaken measurement of APPs in chickens using commercially acquired non-specific, usually human ELISA kits that have not been tested or validated in chickens, and the results of these studies

should be treated with caution. Table 1.3 describes the reported methods by which chicken APPs have been quantified.

APP	Measurement
Cp	Indirectly measured using p-phenylenediamine (PPD) oxidase activity (Sunderman and Nomoto 1970; Martínez-Subiela *et al.*, 2007).
Fb	Heat precipitation method (Thrall *et al.*, 2004)
Fn	ELISA (Lynagh *et al.*, 2000)
Ovt	ELISA (Xie *et al.*, 2002b; Rath *et al.*, 2009)
PIT54	Commercially available kits for measuring haptoglobin are based on the haemoglobin binding activity (Eckersall *et al.*, 1999) are effective with chicken PIT54.
Hpx	Rocket gel electrophoresis using a rabbit anti-chicken hemopexin (Adler *et al.*, 2001).
SAA	Mouse SAA antibody solid phase sandwich ELISA available from Tridelta (Ireland) (Nazifi *et al.*, 2010).
MBL	Sandwich ELISA (Laursen *et al.*, 1998).
AGP	Specific chicken single immunodiffusion test kit available using anti-chicken AGP antibodies of rabbit origin (ECOS Institute, Miyagi, Japan).

Table 1.3: Reported methods of quantifying chicken APP.

More recently APPs have been used as a physiological marker for health and welfare in chickens. In different egg laying and broiler production systems, differences in APP concentrations were compared. The APP AGP was significantly different between egg laying systems (Salamano *et al.*, 2010) and significantly higher in organic broilers compared to conventionally produced broilers, this was attributed to restriction of prophylactic medicines coupled with access to the outside (Tuyttens *et al.*, 2008). Higher stocking density have also been found to increase serum concentrations of APPs (Shakeri *et al.*, 2014; Najafi *et al.*, 2015). Restricted feeding in female broilers however did not affect the serum concentration of APPs (Najafi *et al.*, 2015).

1.2 The Poultry industry

Consumer demand for safe, healthy, competitively priced and readily available meat has resulted in a massive increase in the popularity of poultry meat over the last 50 years. The poultry industry has changed from small scale production to large scale units able to produce the large volume required to meet the growing consumer appetite for chicken. In 2014 109,000 metric tons of poultry meat was produced worldwide, and this figure is projected to reach 111,000 for 2015 (WattAgNet, 2016). Advances in animal breeding and genetic selection have facilitated the advancement in scale of production of the broiler chicken (*Gallus gallus*) along with advances in nutrition, management and disease control. Through these advancements there have been improvements in feed conversion ratio (FCR) allowing for improved growth rates and an increase in breast yield. Selective breeding for increased body weight in broilers has brought about a reduction in the time required for a bird to reach processing weight. In 1953, 10.5 weeks were required for a broiler to reach a final live weight of 1.45 kg; however, by 2001 birds were reaching 2.67 kg in only 6 weeks (Swaggerty *et al.*, 2009).

Selection based heavily on growth characteristics and other phenotypic traits is reported to adversely affect immune competence, leaving chickens and turkeys more susceptible to disease (Swaggerty *et al.*, 2009). Experimental genetic selection of poultry based on immunity or inherent resistance to a specific infectious agent, such as Marek's disease virus or avian leukosis virus, or to a vaccine, resulted in chickens resistant to specific pathogens with either poor growth characteristics or a poor response to other pathogens or vaccines (Zekarias *et al.*, 2002; Swaggerty *et al.*, 2009). As such broiler breeding companies approach the selection of the modern broiler chicken holistically where performance traits are combined with those associated with the health and robustness of the bird. The increased statistical and genomics technologies have enabled breeding programmes to evolve from selection for 'simple' and 'single' criteria to selection for multiple trait criteria that balance production, reproduction, product quality, and animal robustness characteristics to neutralise possible antagonistic effects (Neeteson, 2010). In order to achieve this balanced breeding broiler selection is through multi-environment selection and the inclusion of liveability and robustness traits within the selection process.

As poultry production continues to grow, infectious diseases remain a threat to the industry. Pathogens have the potential to spread and cause a rapid onset of disease, owing to the large homogeneous populations housed in close confinement. Sub-clinical disease is also detrimental, effecting FCRs, growth rates and negatively affecting productivity. As well as impacting negatively on production, disease is also detrimental to bird welfare. Historically antimicrobials were at the forefront of disease control in poultry production. Concern about the overuse of antimicrobials and the subsequent ban on the use of antimicrobial growth promoters in the EU, together with the threat of exotic and emerging diseases mean that there is a need to identify and implement alternatives to disease control (EC Regulation No. 1831/2003; Kogut, 2009). Focusing on the immune system of the bird is one of a number of approaches that can be taken to address these issues.

Using immune parameters such as antibody production, lymphocyte proliferation, phagocyte activity or parasite load to describe immune responsiveness that is correlated with resistance or susceptibility to a pathogen (Zekarias, *et al*. 2002) allows measurable immune parameters to be applied to the selection of poultry. The IIR is non-specific, directs the AIR and has the ability to be augmented and stimulated. This makes the APR an appropriate target for research into immune competence in chicken with the aim of selecting poultry with an efficient innate immune response (Kogut, 2009; Swaggerty *et al.*, 2009). Studies, undertaken with the aim of selecting poultry based on an effective and efficient IIR to produce a population of birds that have multivalent protection against diverse pathogens and increased livability in the field, have revealed that cytokine mRNA expression by heterophils, may be a useful selection tool (Swaggerty *et al.*, 2006). *In vitro* heterophil function corresponds with increased *in vivo* resistance to Gram-positive and Gram-negative bacterial infections and there are increased mRNA expression levels of pro-inflammatory cytokines/chemokines in heterophils isolated from resistant lines compared to susceptible lines. Measurable differences in innate responsiveness are under genetic control with strong pro-inflammatory cytokine and chemokine responses associated with increased resistance against disease (Swaggerty *et al.*, 2009).

The inducible APR can also be evaluated by measuring serum APPs. The changes in the APP profile of an animal during an APR represent an immune parameter by which the degree of immune competence could be measured (Kogut, 2009). While many studies have detailed APPs in chickens, it is only the APP mannan binding lectin (MBL) that has been evaluated as a selection parameter. Mannan binding lectin belongs to a group of proteins called collectins which, upon recognition of infectious agents, activates a number of cellular defence mechanisms including phagocytosis, modulation of cytokines and immunoglobulin secretion (Nielsen *et al.*, 1999; Juul-Madsen *et al.*, 2003; Schou *et al.*, 2010). Although classed as a minor acute phase reactant, MBL plays an active and prominent role in the immune response of chickens (Nielsen *et al.*, 1998b) and expression of MBL has been shown to be genetically influenced. Selecting two lines of chickens for low and high levels of serum MBL concentration resulted in significantly higher MBL levels in the high line and lower MBL levels in the low line by the 6^{th} generation. When experimentally infected with IB virus, there was a 1.6 fold and 2.3 fold increase in MBL in the low and high lines respectively (Juul-Madsen *et al.*, 2007).

Although the expression MBL is malleable to genetic selection, high levels of circulating APPs may not necessarily be optimal and desirable, because as effective as the APR is, it is costly in terms of energy expenditure and behavioural changes (Klasing, 2004). Studies in physiological ecology and theoretical immunology often refer to "cost" in terms of resources such as the energy needed to mount an immune response, or trade-offs between immunocompetence and other nutrient requiring functions (Read & Allen, 2000). The systemic APR that accompanies and IIR appears to be the most expensive component of immunity in young chickens (Klasing, 2004)

Quantifying the changes in mass of leukocytes and protective plasma proteins in systemic (non-mucosal) components of adult chickens injected intravenously with dead *E. coli* reveals that individual components of the immune system respond to an infectious challenge at very different rates. The APPs are secreted in large amounts by the liver during the first days of most infections. Quantitatively, the increases in the weight of cells and antibodies due to *E. coli* were dwarfed by the increase in the weight of the liver and APPs. Thus the APR was markedly more costly than the subsequent adaptive response (Iseri &

Klasing, 2013). The production of APPs would therefore be nutritionally costly given that significant amount of nutrients are needed to support their *de novo* synthesis (Klasing, 2004). It has also been demonstrated that the mobilisation of skeletal muscle protein that occurs during immune stress response supplies the necessary amino acid precursors for APP synthesis (Liu *et al.*, 2015) leading to further loss of FCR.

Worldwide, the poultry industry faces numerous challenges to remain sustainable. These include the move to more extensive rearing systems and the potential withdrawal of prophylactic and many therapeutic antibiotics (Kogut, 2009). With the rapid evolution of bacteria, viruses and parasites into more pathogenic or drug resistant forms, this means that other methods to reduce the impact pathogen related diseases have on production and welfare are required. The host immune response to pathogens in the earliest stages of infection is a critical determinant of disease resistance and susceptibility (Swaggerty *et al.*, 2009). The IIR is not pathogen-specific (Kogut, 2009), so studying the early immune response is highly relevant when considering the immunity of poultry. The relatively recent discovery that the IIR directs the acquired response supports efforts to select poultry with an efficient early IIR (Swaggerty *et al.*, 2009).

Moving towards APPs as possible targets of the early IIR in chickens requires robust methods of measurements to evaluate these IIR parameters. Methods of measurement must be repeatable, relatively straight forward and capable of handling large throughput of samples. Commercially available ELISA kits are costly, and often have not been fully validated in chickens. Further work is required to investigate normal concentrations of APPs in commercial flocks. All the published data on chicken APPs are derived from small scale experimental studies. To use APPs as research, diagnostic or selective tools in chickens, more baseline data is required.

It is also relevant that infections with less virulent pathogens affect productivity (Kogut, 2009) and a challenging microbial environment in itself can reduce growth and uniformity. Considering this, determining whether APPs are associated with non-specific poor performance in broilers would give further information on how these plasma proteins change with low level sub-clinical

disease. Taking steps to identify other biomarkers associated with poor performance would also widen the scope of assays available to deliverer information on the immune health and performance of broiler chickens.

1.3 Enzyme Immunoassays

1.3.1 Introduction

Quantitative analytical methods that show antigen–antibody reactions through the colour change obtained by using an enzyme linked conjugate and enzyme substrate and that serve to identify the presence and concentration of molecules in biological fluids are generally called enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) (Aydin, 2015). Today these terms are synonyms, used interchangeably that originate from the two research methods that developed two assay techniques that differed in design but utilised the same principle.

The enzyme immunoassay method was developed in the early 1970s when two groups independently developed the concept of labelling antigens or antibodies with enzymes for the detection and quantification of analytes. The EIA method using the enzyme bound horseradish peroxidise bound to IgG was published (Van Weemen & Schuurs, 1971) and in the same year a second group published details of an enzyme linked immunosorbant assay (ELISA) that utilised alkaline phosphatise as the reporter label (Engvall & Perlmann, 1971). Since then EIAs and ELISAs have become household names for medical laboratories, manufacturers of *in vitro* diagnostic products, regulatory bodies, and external quality assessment and proficiency-testing organizations (Lequin, 2005).

The impact of diagnostic immunoassays on patients, clinicians, and the healthcare system is virtually unsurpassed, the number of analytical and clinical investigations relying on these measurement procedures worldwide is exceedingly large, the numbers of measurements and determinations using immunoassay for routine patient care are astronomical (Lequin, 2005). Prior to the development of EIAs and ELISAs, radioactively labelled antibodies and antigens were used. This required specialised facilities, posed a safety risk for operators and presented a problem of radioactive waste (Lequin, 2005). With the development of solid phase, with the antigen or antibody bound to

microtitre plates, commercialisation of EIAs and ELISAs has ensued and these products continue to dominate the diagnostics market. Immunoassay are readily available for the detection of a wide range of biomarkers in veterinary species where they are used both diagnostically and in research environments.

1.3.2 Enzyme immunoassay (EIA) / Enzyme linked immunosorbant assay (ELISA) types

Either the antigen or the antibody is sound to the solid phase, which is typically a 96 well microtitre made of rigid polystyrene, polyvinyl and polypropylene. The microplates used must be able to appropriately adsorb the antigen and the antibody, but not adsorb the components in the other phases (Aydin, 2015). Figure 1.1 details an example well for each type of ELISA and how it produces a result. For a direct ELISA, the antigen is coated to the plate and an enzyme linked antibody used for detection. Indirect ELISAs utilise two antibodies: a primary (unbound) antibody that binds with the antigen coated on the plate to form a complex and a secondary enzyme that binds the complex. This secondary antibody is raised against the same host the primary antibody was produced in and is typically an anti-IgG, antibody. This will bind to the primary antibody, but not the antigen or other analytes from the sample. A sandwich ELISA has the antibody coated to the plate and the antigen will bind to this. Antibody with the detection enzyme bound is then added and this will bind to the bound antigen. In a competitive ELISA, antigen in the form of the sample is added to a well that is coated with an antigen, specific to the same antibody. On addition of the antibody which is labelled, antibody bound to the sample antigen will be washed away, whereas antibody bound to the antigen on the plate will remain and be detectable. Direct ELISAs are reported to have the lowest sensitivity, with false positives an issue. Both indirect and competitive ELISA are highly sensitive and sandwich ELISAs are reported to have the highest sensitivity, 2–5 times more sensitive than all other ELISAs (Aydin, 2015).

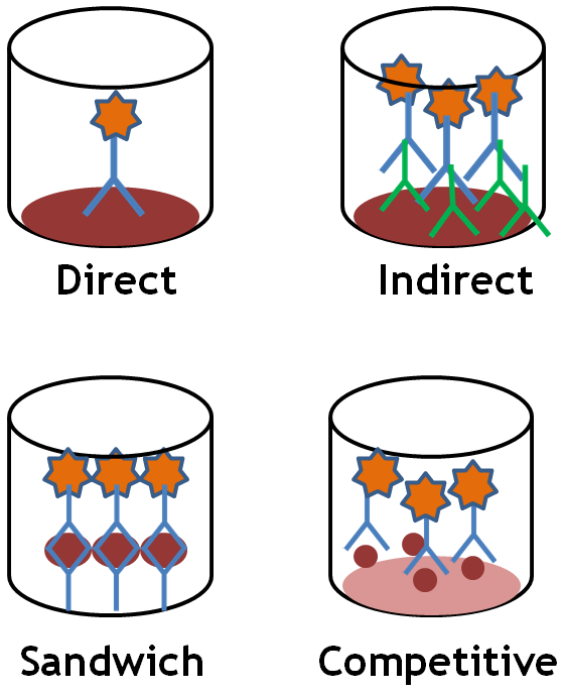


Figure 1.1: Types of ELISAs

1.4 Proteomics

1.4.1 Introduction

A proteome is the protein products of the genome, cell or tissue type (Wasinger *et al.*, 1995) and proteomics the large-scale study of proteins, including their structures and functions (Ceciliani *et al.*, 2014). Since the 1990s, the field of proteomics has become an intense area of research, quantitating the changes in protein expression levels and applying this to drug discovery, diagnostics and therapy (Westermeier *et al.*, 2008). Studying disease at the protein level is challenging and proteins, as mediators of phenotype, require complete understanding if the molecular mechanisms of disease are to be fully understood (Friedman & Lilley, 2008). Proteomics has emerged as a field of research in less than two decades and has developed rapidly, driven by improvements in technology and by the need for analytic approaches that can deliver global protein characterization (Ceciliani *et al.*, 2014).

Mass spectrometry (MS) is one important analytical technique used in proteomics, measuring the mass-to-charge ratio (m/z) of ions based on their

motion or magnetic field (Westermeier *et al.*, 2008). Proteins undergo trypsin digestion, whereby peptide bonds at the carboxyl-terminal of lysine and arginine amino acid residues are cleaved, the peptide masses determined using the MS and a unique peptide mass fingerprint created that make it possible to infer the amino acid composition of those peptides (Lilley & Friedman, 2004). Currently, two major MS platforms are employed for proteomics, differentiated by the mechanism through which ions are generated: these ion sources are termed matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) (Ceciliani *et al.*, 2014).

The complexity of the proteome has made developing methods for efficient separation and sensitive detection of proteins a critical component of this effort. Continued advances in MS technology have enabled the detection of proteins with much greater speed and sensitivity than previously possible (Issaq & Veenstra, 2008). To characterise proteins, the number that the MS is asked to analyze must be temporally limited and this is done by applying either electrophoretic or chromatographic fractionation. By spreading out the proteome, more proteins will ultimately be analyzed within an individual experiment (Issaq & Veenstra, 2008; Ceciliani *et al.*, 2014). Combinations of electrophoresis and chromatography are among the most efficient fractionation systems. Generally, proteins are initially fractionated by electrophoresis, trypsinised to generate peptides that can be further fractionated by high-performance liquid chromatography (LC) before analysis by an ESI-MS that is fed directly with the chromatography eluate (LC-MS). The MS will generally be capable of recording the mass of analytes and be able to isolate and fragment peptide ions (MS/MS, or tandem MS) to generate information about structure. The resulting data are fed to a search engine, such as Mascot (Matrix Science Ltd), which generates *in silico* MS data for the specified genome sequence database and looks for statistically significant matches with the experimentally generated MS data. The data output is a list of potential matches, ranked by confidence, to proteins that may be components of the sample (Ceciliani *et al.*, 2014). Although these efforts can result in the separation and identification of thousands of proteins, no single method can resolve all the proteins in a proteome due to the large number, concentration dynamic range, proteins present at levels below the threshold of detection and also because some

proteins are refractory to analysis or are not represented in the genome database (Issaq & Veenstra, 2008; Ceciliani *et al.*, 2014). Quantitation in MS is based on isotopic or chemical labels that are introduced in a previous step into the organism, at the cellular level, by labelling the intact proteins or peptides, or performed in a label-free mode that requires high reproducibility of the analyses (Almeida *et al.*, 2015).

The application of proteomics centres on the elucidation of the mechanisms of disease and also the search for biomarkers of disease and potential therapeutic targets (Lilley & Friedman, 2004). Despite the potential, proteomic studies have been limited in animal and veterinary research (Almeida *et al.*, 2015) where biomarkers for disease and further insight into the molecular mechanisms by which pathogens cause disease are sought.

1.4.2 Gel based proteomics

1.4.2.1 Two - dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2DE)

Developed by Laemmli, (1970) to separate and study bacteriophage proteins, gel electrophoresis remains at the forefront protein research, albeit with significant modifications and adaptations that have advanced the separation and resolution of proteins within a complex sample. Polyacrylamide gel electrophoresis has been extremely useful as an analytical tool for the separation and quantification of protein species as has the introduction of sodium dodecyl sulfate (SDS) to overcome the problem of protein solubility (Gordon & Jencks, 1963). The ionic detergent SDS favours amino acid-detergent interactions over amino acid-amino acid interactions, promoting the denaturation of proteins (Abdallah *et al.*, 2012).

An important development in proteomics came when O'Farrell, (1975) introduced 2-dimensional separation of proteins (2DE), which has since evolved to become the workhorse of protein separation and the method of choice for differential protein expression analysis (Abdallah *et al.*, 2012). Proteins first undergo isoelectric focusing (IEF) in the first dimension to separate proteins based on their net charge (pH). The orthogonal second dimension is then used to

further separate proteins based on molecular weight in the presence of denaturing conditions. The resolution power is therefore derived from orthogonally combing two separation methods based on two independent parameters: charge and size (O'Farrell, 1975; Lilley & Friedman, 2004; Unlu & Minden, 2002).

Using an isoelectric focussing in the first dimension and a separation via SDS-PAGE in the second dimension, thousands of protein spots can be separated, visualized and quantified in a single 2DE gel. Comparisons of 2DE gels allows for the detection of protein species that differ between the compared states (Unlu & Minden, 2002), with relative abundances of proteins in related samples such as diseased and control tissues, allowing the classes of proteins that differ between two to be determined (Lilley & Friedman, 2004).

Protein spots of interest are removed from the gel digested, extracted from the gel and identified using MS. With a 2DE approach each proteome snapshot becomes a protein map on which hundreds and even thousands of proteins are fractionated and displayed (Unlu & Minden, 2002) making 2DE a powerful approach for descriptive and comparative proteome analysis. It remains the highest-resolution protein separation approach and is inherently quantitative. The separation of intact proteins by charge and mass can highlight post-translational modifications that would not be evident in 1-dimensional electrophoresis or in peptide-based separations (Ceciliani *et al.*, 2014) .

1.4.2.2 Difference gel electrophoresis (DiGE)

Poor resolution results in some classes of proteins, namely those that are of relatively low abundance, very large, or highly charged, being under-represented when 2DE methods are utilised (Issaq & Veenstra, 2008; Ceciliani *et al.*, 2014). While many advances in software alignment tools have been made, reproducibility remains a limitation for 2DE, as ensuring direct spot-to-spot comparison between two separate gels is challenging (Issaq & Veenstra, 2008). High levels of inherent technical and systematic variability will exist between gels affecting reproducibility of generated protein patterns and spot intensities. Gel-to-gel variations result in difficulties in spot detection, matching, and

ultimately distinguishing biological from technical variance (Arentz *et al.*, 2015). Even if the quantification is very accurate and sensitive using 2DE, the relatively high amount of protein necessary for protein identification as well as multiple experimental steps are the major disadvantages of this technique (Megger *et al.*, 2013).

The development of 2D difference gel electrophoresis (DiGE) in 1997 overcame some of these limitations by labelling proteins within samples with spectrally distinct fluorescent dyes prior to electrophoretic separation. The dyes used are cyanine dyes (DiGE CyDye Fluor Cy™ GE Healthcare) and there are three spectrally distinct CyDyes available: Cy2, Cy3, and Cy5. For samples to be compared quantitatively and the comparison to confer any statistical power a pooled internal standard comprising equal aliquots of every sample within the experiment is labelled with the Cy2 CyDye and ran on all gels. This pooled internal standard is labelled once in bulk to avoid variation. Two samples labelled separately to Cy3 and Cy5 and the pooled internal standard labelled with Cy2 are mixed and co-resolved in the same 2DE gel. The gel is scanned with a fluorescent imager at different wavelengths and each of the samples and the pooled standard is separately visible on the single gel. Having been ran together under identical condition with proteins migrating under the exact conditions the problems of reproducibility are overcome (Unlu & Minden, 2002; Lilley & Friedman, 2004; Westermeier *et al*. 2008; Friedman & Lilley, 2008).

Use of the pooled internal standard ensures all proteins in the sample are represented assisting both inter and intra gel matching. Variation in spot volumes due to gel-specific variation will be the same for each sample in the single gel (Lilley & Friedman, 2004). The imaging software developed for the DiGE system: DeCyder™ (GE Healthcare) is used for quantitative analysis. Measurements are made relative to the Cy2 signal for each resolved protein (Friedman & Lilley, 2008) and as the Cy2 signal should be the same for a given protein across all the different gels because it came from the same bulk mixture, any differences represent gel-to-gel variation, which can be effectively neutralised by normalising all the Cy2 values for a given protein across all the gels (Friedman & Lilley, 2008). DeCyder™ uses a triple co-detection algorithm that allows simultaneous detection of labelled protein spots from images that

arise from the same gel and increases the accuracy in the quantification of standardised abundance. The standardised abundances can then be compared across groups to detect changes in the protein expression (Alban *et al.*, 2003). The use of sufficient replicates and the presence of the same pooled standard present in each gel allows for the application of the students t-test and analysis of variance (ANOVA) statistical analysis despite the samples having been separated on different DiGE gels (Lilley & Friedman, 2004).

1.4.3 The chicken proteome

1.4.3.1 Introduction

Proteomic samples can come from many different and varied sources. Investigations can utilise whole plasma or homogenised tissues from experimental or clinically affected animals. The excellent resolving powers of proteomics mean that sub-cellular proteins can be fractionated and identified to reduce the protein complexity (Lilley & Friedman, 2004). The use of proteomics to investigate disease and identify biomarkers in the field of animal health has gained huge momentum over the last 15 years and in poultry, as the areas of application widen, it has becomes possible to investigate more specific and varied areas of poultry health. Comparing the number of publications listed in Web of Science that contain the words ‘proteome’ or ‘proteomics’ and ‘chicken’ or ‘broiler’ or ‘hen’ in their title reveals that the study of chicken proteomes has grown steadily over the last 15 years. Figure 1.2 details this trend.

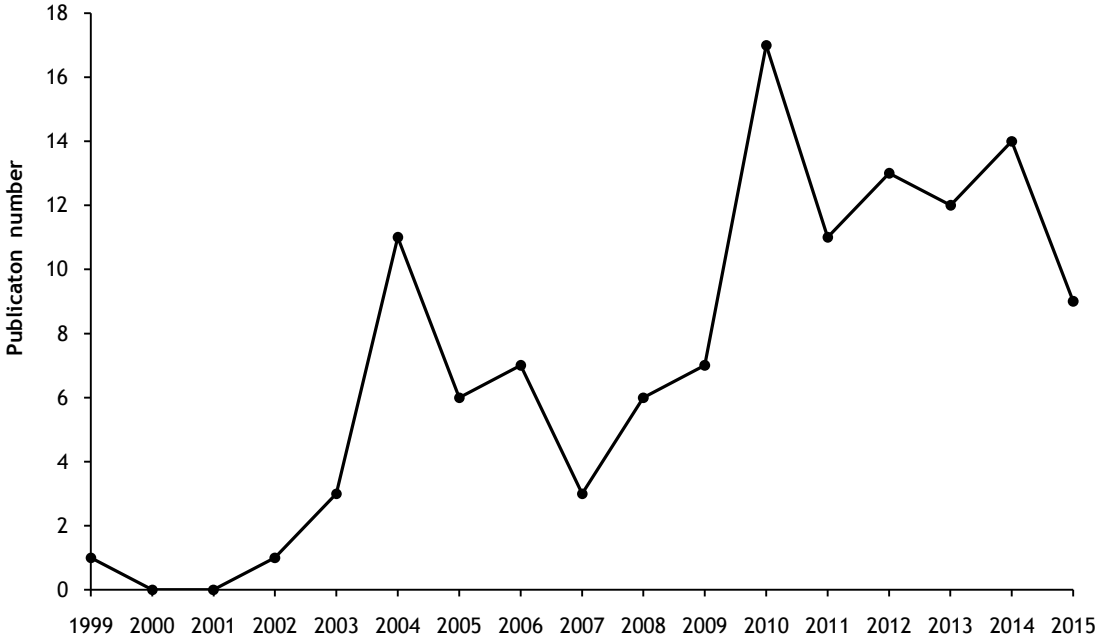


Figure 1.2: The number of publications listed in Web of Science that contain the words 'proteome' or 'proteomics' and 'chicken' or 'broiler' or 'hen' in their title from 1999 to 2015. No publications are listed prior to 1999.

1.4.3.2 The effect of selection on the chicken proteome

Detailing the proteomic differences between genetic lines of chickens which exhibit differential resistance and/or susceptibility to different diseases can reveal molecular mechanisms by which pathogens cause disease in their host and can reveal how a bird's immune system can affect a more efficacious response. One of the few research papers to compare protein expression between different broiler lines found that the serum proteome of two lines challenged orally with *Eimeria (E.) acervulina*, *E. maxima* or *E. tenella* identified a number of differentially expressed proteins. Several metabolic enzymes were identified as potential candidates for early diagnostic markers of *E. acervulina* infection including malate dehydrogenase 2, NADH dehydrogenase 1 alpha subcomplex 9, and ATP synthase. These proteins were detected only in Line A birds that were inoculated with *E. acervulina*. Increases of a protein in response to infection were detected mainly in *E. acervulina* and *E. tenella* infected birds in both lines (Gilbert *et al.*, 2011).

Comparing the proteomes of broiler breeds with different growth profiles can also elucidate to the molecular mechanisms by which some birds effectively

utilise and metabolise the nutrients available to them. Comparing the embryonic liver proteome of two breeds of broiler with different growth and fat deposition characteristics, identified thirty-seven differentially expressed proteins. The results suggest that lipid metabolic proteins and enzymes are inherent characteristics that contribute to the apparent differences in fat deposition between the two strains (Huang, *et al.*, 2010).

1.4.3.3 The chicken intestinal proteome

The intestinal proteome has been a particular popular target in chicken proteomic studies owing to the importance of intestinal health in broiler chickens. Studies of the chicken intestinal proteome have found that proteins in the brush border involved in digestive function, maintenance of membrane potential, membrane trafficking and cytoskeletal organisation changed from day of hatch to 14 days post hatch over time and differed between genetic lines (Gilbert, *et. al.*, 2010). Bacteriocins were found to trigger differential expression of metabolic-related proteins within the jejuna, many of which had antioxidant functions (Wang *et al.*, 2013). The feeding of the probiotic *Enterococcus faecium* (*E. faecium*), differentially expressed forty-two intestinal mucosal proteins, four related to intestinal structure and seventeen related to immune and antioxidant systems of which six were abundant in the broilers fed *E. faecium*, which when taken with the other findings in this study indicated that the chickens employed less nutrients and energy to deal with immune and antioxidant stresses (Luo *et al.*, 2013). The jejunal proteome sampled from 12 to 22 days of age showed developmental changes and the introduction of re-used litter at 12 days resulted in the differential expression of a number of proteins. Many of these proteins were cytoskeletal proteins, particularly actin. Villin-1, an actin associated protein involved in apoptotic processes was shown to be reduced in expression in the challenged birds indicating that many of the changes in cytoskeletal protein expression in the challenged birds were as a result of an increased rate of apoptosis. A number of heat shock proteins were found to decrease in expression over time in the intestine and this was more pronounced in the challenged birds (O'Reilly, 2013). High atmospheric ammonia exposure was found to differentially express forty-three intestinal mucosal proteins, up-regulating proteins related to oxidative phosphorylation and apoptosis and down-regulating proteins related to cell structure and growth,

transcriptional and translational regulation, immune response, oxidative stress and nutrient metabolism; indicating that exposure to ammonia triggered oxidative stress and interfered with nutrient absorption and immune function in the small intestinal mucosa of broilers (Zhang *et al.*, 2015a). The results of these intestinal proteome studies in chickens are comparable with similar studies in other species and indicate that many of the responses to infection and inflammation in the small intestine are conserved among vertebrates.

1.4.3.4 The chicken proteome during disease

Proteomics has been applied to the study of a number of poultry diseases. Studying the splenic proteome over time as a result of Marek's disease infection in susceptible and resistant chickens revealed a number of differentially expressed proteins included antioxidants, molecular chaperones, proteins involved in the formation of cytoskeleton, protein degradation and antigen presentation, signal transduction, protein translation and elongation, RNA processing and cell proliferation (Thanthrige-Don *et al.*, 2010). The peripheral blood mononuclear cell proteome during Newcastle disease virus infection revealed thirty-five proteins that consistently showed differential expression. These proteins functioned in macromolecular biosynthesis, cytoskeleton organization, metabolism, stress responses, and signal transduction (Deng *et al.*, 2014). The proteomic changes induced in host cells infected with avian reovirus (ARV), the etiological agent of several diseases, revealed the majority of protein expression changes occurred 72 hours post-infection and that fifty-one proteins had differential expression levels. All identified proteins involved in signal transduction, RNA processing, and the ubiquitin-proteasome pathway were downregulated in infected cells, whereas proteins involved in DNA synthesis, apoptosis, and energy production pathways were upregulated (Chen *et al.*, 2014). Metabolic diseases have also been the focus of proteomic studies with the hepatic proteome of broilers afflicted with ascites syndrome compared to their normal counterparts (Wang et al., 2012) and the protein extracts of cartilage from normal and tibial dyschondroplasia (TD) affected growth plates compared (Rasaputra *et al.*, 2010).

1.4.3.5 Egg, meat and reproductive proteomes

Proteomic methods are also readily applicable to poultry meat and egg quality with a number of studies investigating the proteome of muscle (Doherty *et al.*, 2004), carcass characteristics and meat quality (Zheng *et al.*, 2014). Proteomic-based methods have also been developed for the detection of chicken meat within mixed meat preparations (Sentandreu *et al.*, 2010). The area of egg and egg shell research is undoubtedly the area of poultry research that has utilised proteomic technologies the most with a significant number of proteomic studies detailing the egg proteome and the egg shell/cuticle proteome. The eggshell is a barrier that plays an important role in the defence of the egg against microbial and other infections and it protects the developing bird against unfavourable impacts of the environment and is essential for the successful reproduction of birds. The extractable proteins of the eggshell matrix have been studied extensively and many of them have been identified (Mikšík *et al.*, 2014). In other reproductive related areas semen quality has been assessed using proteomics (Labas *et al.*, 2015) and the proteomic analysis of the muscularis complexus (the pipping muscle) in broiler chicken embryos has also been undertaken (Sokale *et al.*, 2012).

1.4.3.6 Other tissue proteomes in chickens

The effects of heat stress (Tang *et al.*, 2015) and ammonia toxicity (Zhang *et al.*, 2015b) on the hepatic proteome have been investigated. Proteomic approaches have also been undertaken to study heart muscle development, studying the proteome of different heart structures at three stages of chicken embryonic development (Bon *et al.*, 2010). The proteome of brain tissue form chickens infected by the neurovirulent avian influenza virus have also been detailed (Zou *et al.*, 2010). The proteome of the bursa of Fabricius, an important gut associated lymphoid tissue where chicken B-cells develop has been detailed during embryonic and post-hatch development. One hundred and fifty three protein spots with significantly different intensities were identified with several proteins assigned to retinoic acid metabolism (e.g. retinal-binding protein 5) and the actin-cytoskeleton (e.g. vinculin and gelsolin) (Korte *et al.*, 2013).

1.5 Aims of study

In veterinary species there is increasing emphasis on the identification of biomarkers for disease resistance, enhanced immunity and optimal vaccine response. Susceptibility to infection, sub-clinical disease and the emergence of drug resistant pathogens are major issues in poultry production and impact negatively on health, welfare and overall productivity making this is a timely and appropriate target for novel biomarker investigation. Acute phase proteins are widely used as disease biomarkers and these are an appropriate target on which to focus a study owing to their role in early IIR. Despite the number of publications on APPs in chickens, the variety of the research is low. Often the same APPs are studied and the immunogen LPS is typically used to initiate an APR. There are many APPs have not yet been confirmed as acute phase responders in chickens and there is a lack of reliable, specific and economically viable assays and the availability of commercial ELISA is limited. This in itself has somewhat distorted the research publications with many APPs not detailed at all and others over represented in the literature. Developing new immunoassays will broaden the spectrum of APPs measured in chickens.

The development of immunoassays is lengthy and costly and to circumvent this a quantitative proteomic approach will allow the identification and measurement of a large number of proteins within a single series of experiments. For APPs to be used diagnostically or as a selective tool for breeding more baseline data is required on normal APP concentrations and on how baseline concentrations change on commercial farms. Looking at the APP concentrations from normal and culled chickens will give comparative data and contextualise other results.

It is the further aim of this work to detail novel biomarkers of the APR that may not have been considered previously as such. Plasma biomarkers for intestinal health will also be studied. Acute phase proteins will be measured to determine whether an intestinal challenge, in the form of re-used litter, induces an APR. As a result of initial studies, the protein apolipoprotein AIV will be investigated and an ELISA developed. The application of APP measurement to a number of areas of poultry science is possible, but without robust and validated methods of measurement that are economical and capable of a high throughput of samples, the full extent to which these biomarkers can be utilised will not be recognised.

1.6 Thesis outline

Chapter 2 measures the APPs Ovt, PIT54 and ceruloplasmin (Cp) in three separate studies. The first investigates Cp and PIT54 concentrations from broilers from a commercial unit. These broilers have been culled due to stunting or lameness or are healthy. The aim of this study was to look at the baseline data for these APPs and determine whether there was any difference between seemingly healthy broilers and culled birds from a normal production environment. In a second study APP concentrations are measured in broilers with experimental infections of *E.tenella*, *E.coli* and combinded *E.tenella* and *E.coli* infections. A 1DE gel is also undertaken to survey the proteins present. The third study compared the APP concentrations of broilers with different gait scores and also details the plasma proteome of using 2DE.

Given the poor reproducibility of the Ovt assay described and used in Chapter 2, **Chapter 3** sought to develop another Ovt assay utilising an antibody raised against egg derived Ovt. This chapter details the development of the assay and its validation.

With the aim of widening the scope of this study to APPs that have not been previously detailed in the literature and investigating novel biomarkers of the APR in chickens, quantitative proteomics was utilised in **Chapter 4** to determine the relative quantification of a number of APPs and proteins that appear to be differentially expressed between three different acute phase groups. Three pools were established initially, these were highly acute phase (HAP), acute phase (AP) and non-acute phase (NAP) pools. These pools underwent proteomic shotgun analysis and proteins found to be differentially expressed between the pools were identified and those with highest fold changes were selected for further measurement on individual samples. It was the aim of this Chapter to identify and measure the APPs serum amyloid A (SAA) and C-reactive protein (CRP) in chickens and a number of enrichment steps, namely molecular weight fractionation and affinity chromatography, were undertaken to aid in the identification and measurement of these APPs.

Chapter 5 uses the same proteomic approach as Chapter 4 to measure the protein apolipoprotein AIV (apo-AIV), a protein reported to have various functions including APP and a satiety protein. A further protein gelsolin, an actin

associated protein was also measured. An ELISA to measure apoAIV was developed and validated. A study to investigate the APR as a result of a microbial challenge, delivered in the form of reused litter was undertaken and the APPs determined by both ELISA and quantitative proteomics. The protein villin, found in a previous study to be a possible biomarker of intestinal health (O'Reilly, 2013), was investigated using western blotting.

Chapter 6 utilised DiGE technology to compare the tendon proteomes of broiler chickens with ruptured gastrocnemius tendons (GT). The aim of this work was to detail the GT proteome in chickens and identify differentially expressed proteins, which could elucidate to the pathophysiology of this condition.

Chapter 7 summarises the work of the whole thesis and details areas in which improvement could be made and recommendations for future work.

Chapter 2

Acute phase protein analysis in broiler chickens

2.1 Introduction

A series of studies were utilised to investigate ceruloplasmin (Cp), PIT54 and ovotransferrin (Ovt) serum concentrations in broiler chickens. These APPs are the most widely measured APPs detailed in the literature. It is the aim of this chapter to determine whether broiler chickens, culled on farms due to lameness and stunting had higher APP concentrations than their healthy counterparts. Measuring APPs in healthy birds from commercial farms would aid in the establishment of baseline concentrations and reveal the normal variation among healthy birds. Detailing the APPs in culled birds would also reveal whether culled birds from commercial farms exhibit an APR at culling, and this may provide information as to the cause of non-specific conditions for which the birds were culled.

Two further studies focus on lameness and Coccidiosis, two diseases that are widespread and cause significant financial loss to the poultry industry. Measuring APPs and utilising proteomic methods will gain further insight into the acute phases of these diseases. The APPs Cp, PIT54 and Ovt will be described in further detail in the following sections, being the APPs this chapter focuses its attentions. Other APPs will be introduced in more detail in the following chapters where work on these APPs is described.

2.1.1 Ceruloplasmin

Ceruloplasmin (Cp) is a vertebrate plasma protein belonging to the multicopper oxidase family, a family characterised by their different copper sites (Floris *et al*. 2000). Ceruloplasmin is the only copper oxidase to occur in animal species (Calabrese *et al*. 1988) and contains over 95% of the copper found in the plasma (Martinez-Subiela *et al*. 2007). Avian blood is very low in copper compared to mammals and as such has lower serum levels of Cp (Disilvestro and Harris, 1985). Human serum contains approximately 30mg Cp per 100ml serum whereas chicken serum has 3mg Cp per 100ml (Disilvestro and Harris, 1985).

2.1.1.1 Ceruloplasmin structure and function

Ceruloplasmin is a multifunctional protein both storing and transporting copper within the body. Ceruloplasmin donates copper in a regulatory fashion with evidence of Cp receptors on the membranes of numerous different cell types,

supporting Cp's role as a cell signalling multifunctional enzyme within many different metabolic pathways (Floris *et al.,* 2000). Ceruloplasmin is an antioxidant protein and historically preventing iron induced oxidative stress has been considered the principle role of the protein *in vivo;* as ferroxidation by Cp is a physiologically crucial event with Cp able to oxidise the divalent iron Fe (II), efficiently preventing iron induced oxidative stress (Floris *et al.,* 2000). While Cp has been long recognised as a plasma protein synthesised by hepatocytes, there is evidence in other species to indicate that other cell types also synthesis Cp (Floris *et al* 2000).

2.1.1.2 Ceruloplasmin and the acute phase response in chickens

The first paper to describe Cp as an APP in chickens was Butler, *et al.* (1972) who noted that a single injection of endotoxin from *E. coli* increased serum Cp concentration by 2 – 5 fold, the degree of increase varying with the strain of *E. coli* used. The maximal increase in Cp was 24 hours post injection and increased levels were still detectable 48 hours after injection. Further studies have also described Cp as a positive acute phase reactant. *Salmonella* serotype Gallinarum delivered *per os* into the crop of chickens, showed Cp to increase the quickest of all the APPs measured in this study, with peak serum Cp levels measured 3 – 5 days post infection. Serum concentration then progressively decreased (Garcia *et al.,* 2009).

Serum CP increased significantly in response to *Eimeria (E.) tenella* infection (Richards and Augustine, 1988; Georgieva *et al.,* 2010) and combined *E. coli* and *E. tenella* infection (Georgieva *et al.,* 2010). Infection with *Eimeria (E.) acervulina* did not significantly increase Cp concentrations (Richards and Augustine, 1988). Conversely chickens infected with infectious bursal disease virus (IBDV) had decreased serum Cp concentration, with the decrease even more pronounced in the most severely infected birds (Nazifi *et al.,* 2010). Further work would be needed to confirm whether, during viral or IBDV infections Cp is indeed a negative APP.

2.1.1.3 Quantification of Ceruloplasmin in chickens

Disilvestro and Harris (1985) characterised chicken Cp using SDS PAGE and revealed Cp to have a molecular weight of 130kDa, a slightly smaller band was

also present, which the authors suggested could be minor fragments of the main component. Calabrese *et al*, (1988), found chicken Cp to have a higher molecular weight of approximately 140kDa. Disilvestro and Harris (1985) produced chicken Cp antibodies for immunoelectrophoresis and yielded a single band corresponding to Cp on western blots. Serum Cp concentration can be measured indirectly by measurement of the p-phenylenediamine (PPD) oxidase activity. Cp catalyses the oxidation of PPD to yield a purple coloured product whose rate of formation can be determined by spectrophotometry. The rate of formation of the coloured oxidation product is proportional to the concentration of serum Cp, if a correction is made for non enzymatic oxidation of PPD (Sunderman and Nomoto, 1970). The oxidase activity of chicken Cp assayed between pH 6 – 8 was found by Calabrese *et al*. (1988) to be five times higher than the value measured for mammalian Cp under the same experimental conditions with maximum at around pH 6.8. The PPD method is the most widely cited method for measuring Cp.

2.1.2 PIT54

Under certain pathological conditions such as infection the red blood cell (RBC) (erythrocyte) membrane can destabilise resulting in the lyses of the cell and leakage of haemoglobin (Hb) into circulation (Wicher and Fries, 2006). As evolution made Hb more efficient as an oxygen carrier within the RBC, the protein appears to have developed harmful properties outside the cell. Haemoglobin outside the cell is toxic, proinflammatory and pro-oxidative causing damage to cells and tissues and haem associated iron will support microbial growth (Ceron *et al*. 2005; Wicher and Fries, 2010). Plasma proteins have therefore evolved that bind free Hb or its haem group, blocking its detrimental effects and mediating its removal from the blood stream (Wicher and Fries, 2010). Haemoglobin and haem scavenging in plasma involves a number of different proteins: haptoglobin (Hp), high and low density lipoproteins (HDL, LDL), serum albumin, Hpx, α-1 microglobulin (Ascenzi *et al*., 2005) and an avian species named PIT54 (Wicher and Fries, 2006).

Historically the Hb binding protein in chickens was considered to be Hp or a haptoglobin-like protein until analysis of the Hb binding protein in chickens found that it binds avian and reptilian Hb but not mammalian Hb. In contrast

human Hp binds both chicken and human Hb with great affinity (Musquera *et al.*, 1979). A report by Delers *et al.*, (1983) analysing mammalian Hp and the Hb binding protein in chickens revealed significant structural differences between the two proteins. Detailed analysis of the chicken genome found there to be no gene coding a protein similar to mammalian Hp. Instead the protein referred to as PIT54 was identified, which fulfils a similar role in chickens as Hp does in mammals (Iwasaki *et al.*, 2001; Wicher and Fries, 2006). PIT54 is found only in avian species having been identified in geese, ostriches and emus. Although absent in chickens, Hp is found in paleognathus birds (ostriches and emus) where Hp has been conserved alongside PIT54 (Wicher and Fries, 2010). Despite the evidence, some of the literature still refers to chicken Hp as opposed to PIT54. Given the structural and functional similarities between the two proteins the following section details work conducted on both Hp and PIT54.

2.1.2.1 Haptoglobin and PIT54 structure and function

PIT54 is a soluble member of the family of scavenger receptor cysteine rich proteins (Wicher and Fries, 2006). Structurally PIT54 has been demonstrated to have three glycosylation variants that differ in their molecular weight and structure, though distribution of the glycosylation variants are not altered during an APR when induced in layer chickens with turpentine (Delers *et al*. 1988). Haptoglobin is an anti-inflammatory protein modulating prostaglandin synthesis and inhibiting granulocyte chemotaxis and phagocytosis (Ceron *et al.*, 2005). PIT54 too has been shown to have potent antioxidant properties, inhibiting superoxide production by phagocytes, thus inhibiting the overproduction of reactive oxygen species that cause oxidative damage (Iwasaki *et al*. 2001). This supports the notion that PIT54 evolved from an anti-inflammatory protein which evolved Hb binding ability and, like Hp, retains these anti-inflammatory properties (Wicher and Fries, 2010).

PIT54 and Hp are antibacterial proteins, binding free haemoglobin rendering it unavailable for bacterial growth. Haemoglobin iron availability and the susceptibility to infection are intimately linked with pathological iron overload predisposing to bacterial infection (Ascenzi *et al.*, 2005). Haptoglobin also has a role in wound healing binding Hb in infected areas to prevent bacterial growth and sepsis and this may account for Hp expression in adipose tissue (Ceron *et*

al., 2005). Free Hb in systemic circulation will pass through the glomerular filter, causing renal damage. When Hp forms a soluble complex with Hb in plasma they are not filtered through the glomeruli but delivered to the reticuloendothelial system and after internalisation into liver parenchymal cells the complex dissociates into two subunits that are subsequently degraded (Langlois and Delanghe, 1996; Ascenzi *et al.*, 2005) and the iron is reused.

2.1.2.2 PIT54 and the acute phase response in chickens

Haptoglobin is a positive APP and serum concentrations will increase during infection, inflammation and trauma and tissue damage. Measuring the serum concentration of Hp provides valuable diagnostic information in cattle and sheep where normal Hp values are <0.1mg/ml but increase 100 fold or more in response to infection, making Hp a major APP in these species. In dogs, cats and pigs more modest increases (2 – 5 fold) occur but these increases are still diagnostic (Eckersall *et al.,* 1999).

In chickens Nazifi *et al.*, (2010, 2011) using a commercial Hp kit based on Hb binding affinity that also measures the concentration of PIT54, found that chickens had no significant increase in PIT54 during IBDV infection, yet had a significant increase as a result of infectious bronchitis (IB) infection.

Investigating the effect of bacterial challenge on PIT54 serum concentrations Garcia *et al.*, (2009) found that intra-crop inoculation of *Salmonella enterica*, serovar Gallinarum, resulted in a significant increase throughout the course of the 10 day challenge. As a result of parenteral administration of *S. Typhimurium* LPS, mean PIT54 serum concentrations significantly increased 1.5 fold (Millet *et al.*, 2007). Georgieva *et al.*, (2010) compared serum PIT54 after experimental infection with *E. coli, E. tenella* and combined infection of both pathogens. The increase in PIT54 was highly significant as a result of *E. coli* infections and combined infections, however serum PIT54 levels did not increase significantly as a result of *E. tenella* infection alone. The reported increases in PIT54 during an APR would classify it as a minor APP in chickens.

2.1.2.3 Quantification of PIT54 in chickens

Owing to the functional similarities between PIT54 and Hp, a commercially available assay based on the biochemical activity of Hp will work with PIT54

from chicken serum (Georgieva *et al.*, 2010). For example Nazifi *et al.* (2010) measured PIT54 according to the preservation of the peroxidise activity of Hb which is directly proportional to the Hp (or PIT54) if using for chickens (Tridelta, Wicklow, Ireland).

2.1.3 Ovotransferrin

The transferrins are an extended family of metal binding transport proteins with an *in vivo* preference for binding ferric iron. They are single chain glycoproteins and are widely distributed in physiological fluids and cells of vertebrates (Aguilera *et al.*, 2003). Mammals have serum transferrin, synthesised in the liver and lactoferrin a major milk protein, synthesised in the mammary gland. Melanotransferrin has also been identified in malignant melanoma cells (Aguilera *et al.*, 2003). Chickens have neither transferrin or lactoferrin, instead synthesising ovotransferrin (Ovt) in the liver in response to proinflammatory cytokines and in the oviduct under the influence of oestrogen (Hallquist and Klasing, 1994). Historically the word conalbumin was used to describe both the egg and serum transferrin in chickens. Williams (1968) as well as advocating ovotransferrin as a more informative name to describe the egg protein, demonstrated that both serum and egg Ovt were the same protein differing only in their glycosylation. More recently serum transferrin has adopted the 'ovo-' and the word ovotransferrin is widely used to describe both egg and serum transferrin in chickens. Ovotransferrin is a positive APP in chickens (Hallquist and Klasing, 1994; Xie *et al.*, 2002a) with a number of studies confirming that serum Ovt increases during an APR due to a variety of experimentally induced infections and inflammatory processes. This differs from mammals where serum transferrin is a negative APP with expression being down regulated during inflammation (Ceron *et al.*, 2005). Synthesis of egg Ovt however responds in a negative fashion during an APR, with the oviduct magnum decreasing mRNA synthesis for egg Ovt with a resultant cessation to egg laying (Hallquist and Klasing, 1994).

2.1.3.1 Ovotransferrin structure and function

Ovotransferrin shows marked sequence and structural homologies with both mammalian serum transferrin and mammalian lactoferrin (Giansanti *et al.*, 2007). Sequence alignment (Figure 2.1) of chicken Ovt and human

lactotransferrin and serotransferrin shows 40.97% sequence homology between the three transferrin protein sequences. In chickens serum Ovt is synthesised by the liver and released into the blood stream in response to proinflammatory cytokines. Egg Ovt is synthesised by cells in the oviduct under the control of oestrogen. It is predominantly an egg white protein though it has also been found in the yolk, shell membranes and shell of the egg (Stevens, 1991). Ovotransferrin produced in the oviduct is incorporated into the egg white where it has an important antibacterial action on bacteria that enter through the porous egg shell.

Chicken egg Ovt and serum Ovt have been identified as being the same protein that exists in two protein isoforms that have a similar amino acid sequence and differ only in their glycosylation patterns and composition of their carbohydrate moieties (Williams, 1968; Thibodeau *et al.*, 1978; Xie *et al.*, 2002a). The egg white Ovt glycosyl-group is present as a single oligosaccharide composed of residues of mannose and 8 residues of N- acetylglucosamine glycan. Serum Ovt is mostly composed of a single unit of 2 residues of mannose, 2 residues of galactose, 3 residues of N-acetylglucosamine and either 1 or 2 residues of sialic acid (Williams, 1968).

It is hypothesised that in birds, Ovt carries out the biological functions which in mammalian species are split between serum transferrin and lactoferrin. Like mammalian transferrin, serum OVT is involved with iron transport and delivery to cells and egg. Ovt displays many biological functions similar to that of lactoferrin related to innate immunity, carrying out in egg white the same protective antibacterial functions that lactoferrin carries out in mammalian milk (Giansanti *et al.*, 2007). Ovotransferrin, like other members of the transferrin family, is an antibacterial protein, able to sequester, store and transport iron. It is Ovt's iron sequestering function that deprives bacteria of the iron essential for growth (Xie, *et al.*, 2002b) and contributes to its antibacterial action. Ovotransferrin has been demonstrated to have a direct action on Gram negative bacteria as Ovt is able to permeate the outer membrane of *E.coli*, reach the inner membrane and cause selective permeation of ions. This results in dissipation of the electrical potential, which together with a change in membrane potential and alteration in pH, results in uncoupling of the respiration-dependant energy production resulting in bacteriostasis (Aguilera *et*

al., 2003). Transferrins also disrupt the bacterial type III secretion systems which promote bacterial attachment (Van Droogenbroeck, Beeckman *et al.*, 2008). *In vitro* work is highly suggestive of a crucial role of Ovt against viral infection as both isoforms of Ovt (serum and egg white) have been shown to exert antiviral activity towards Marek's disease virus, an avian herpes virus (Giansanti *et al.* 2007).

```
SP|P02789|TRFE_CHICK MKLILCTVLSLGIAAVCFAAPPKSVIRWCTISSPEEKKCNNLRDLT----QQERISLTCV 56
SP|P02788|TRFL_HUMAN MKLVFLVLLFLGALGLCLAGRRR-SVQWCAVSQPEATKCFQWQRNMRK---VRGPPVSCI 56
SP|P02787|TRFE_HUMAN MRLAVGALLVCAVLGLCLAVPDK-TVRWCAVSEHEATKCQSFRDHMKSVIPSDGPSVACV 59
*:* . .:*  .  .:*:*   :  ::**::*. * .** . :            ::*:

SP|P02789|TRFE_CHICK QKATYLDCIKAIANNEADAISLDGGQAFEAGLAPYKLKPIAAEVYEHTEGSTTSYYAVAV 116
SP|P02788|TRFL_HUMAN KRDSPIQCIQAIAENRADAVTLDGGFIYEAGLAPYKLRPVAAEVYGTERQPRTHYYAVAV 116
SP|P02787|TRFE_HUMAN KKASYLDCIRAIAANEADAVTLDAGLVYDAYLAPNNLKPVVAEFYGSKEDPQTFYYAVAV 119
:: : ::**:*** *.***::**.*  ::* *** :*:*:.**.*   .   * ******

SP|P02789|TRFE_CHICK VKKGTEFTVNDLQGKTSCHTGLGRSAGWNIPIGTLLHRGAIEWEGIESGSVEQAVAKFFS 176
SP|P02788|TRFL_HUMAN VKKGGSFQLNELQGLKSCHTGLRRTAGWNVPIGTLRP--FLNWTGP-PEPIEAAVARFFS 173
SP|P02787|TRFE_HUMAN VKKDSGFQMNQLRGKKSCHTGLGRSAGWNIPIGLLY----CDLPEP-RKPLEKAVANFFS 174
***   * :*:*:* .****** *:****:*** *      :        :* ***.***

SP|P02789|TRFE_CHICK ASCVPGATIE--QKLCRQCKGDPKTK--CARNAPYSGYSGAFHCLKDGKGDVAFVKHTTV 232
SP|P02788|TRFL_HUMAN ASCVPGADKGQFPNLCRLCAGTGENKCAFSSQEPYFSYSGAFKCLRDGAGDVAFIRESTV 233
SP|P02787|TRFE_HUMAN GSCAPCADGTDFPQLCQLCPGCG-----CSTLNQYFGYSGAFKCLKDGAGDVAFVKHSTI 229
.**.* *      :**: * *        :    * .*****:**:** *****::.:*:

SP|P02789|TRFE_CHICK NENAPD--QKDEYELLCLDGSRQPVDNYKTCNWARVAAHAVVARDDNK-VEDIWSFLSKA 289
SP|P02788|TRFL_HUMAN FEDLSDEAERDEYELLCPDNTRKPVDKFKDCHLARVPSHAVVARSVNGKEDAIWNLLRQA 293
SP|P02787|TRFE_HUMAN FENLANKADRDQYELLCLDNTRKPVDEYKDCHLAQVPSHTVVARSMGGKEDLIWELLNQA 289
*:  :  ::*:***** * :*:***::* *. *:* :*:****.     : **.:* :*

SP|P02789|TRFE_CHICK QSDFGVDTKSDFHLFGPPGKKDPVLKDLLFKDSAIMLKRVPSLMDSQLYLGFEYYSAIQS 349
SP|P02788|TRFL_HUMAN QEKFGKDKSPKFQLFGSPSG----QKDLLFKDSAIGFSRVPPRIDSGLYLGSGYFTAIQN 349
SP|P02787|TRFE_HUMAN QEHFGKDKSKEFQLFSSPHG-----KDLLFKDSAHGFLKVPPRMDAKMYLGYEYVTAIRN 344
*..** *.. .*:**. *       *********  : :**  :*: :***  * :**:.

SP|P02789|TRFE_CHICK MRKDQLT--PSPRENRIQWCAVGKDEKSKCDRWSVVSNGDVECTVVDETKDCIIKIMKGE 407
SP|P02788|TRFL_HUMAN LRKSEEE--VAARRARVVWCAVGEQELRKCNQWSGLSEGSVTCSSASTTEDCIALVLKGE 407
SP|P02787|TRFE_HUMAN LREGTCPEAPTDECKPVKWCALSHHERLKCDEWSVNSVGKIECVSAETTEDCIAKIMNGE 404
:*:       : .   : ***:...*  **:.**  * *.: *  .. *:***  :::**

SP|P02789|TRFE_CHICK ADAVALDGGLVYTAGVCGLVPVMAERYDDESQCSK----TDERPASYFAVAVA-RKDSNV 462
SP|P02788|TRFL_HUMAN ADAMSLDGGYVYTAGKCGLVPVLAENYKSQQSSDPDPNCVDRPVEGYLAVAVVRRSDTSL 467
SP|P02787|TRFE_HUMAN ADAMSLDGGFVYIAGKCGLVPVLAENYNKS------DNCEDTPEAGYFAIAVVKKSASDL 458
***::**** ** ** ******:**.*...          *    .*:*:**. :. :.:

SP|P02789|TRFE_CHICK NWNNLKGKKSCHTAVGRTAGWVIPMGLIHNRTGTCNFDEYFSEGCAPGSPPNSRLCQLCQ 522
SP|P02788|TRFL_HUMAN TWNSVKGKKSCHTAVDRTAGWNIPMGLLFNQTGSCKFDEYFSQSCAPGSDPRSNLCALCI 527
SP|P02787|TRFE_HUMAN TWDNLKGKKSCHTAVGRTAGWNIPMGLLYNKINHCRFDEFFSEGCAPGSKKDSSLCKLCM 518
.*:.:********** ***** *****:.*:   *.***:**:.*****   * ** **

SP|P02789|TRFE_CHICK GSGGIPPEKCVASSHEKYFGYTGALRCLVE-KGDVAFIQHSTVEENTGGKNKADWAKNLQ 581
SP|P02788|TRFL_HUMAN GDEQ-GENKCVPNSNERYYGYTGAFRCLAENAGDVAFVKDVTVLQNTDGNNNEAWAKDLK 586
SP|P02787|TRFE_HUMAN GS---GLNLCEPNNKEGYYGYTGAFRCLVE-KGDVAFVKHQTVPQNTGGKNPDPWAKNLN 574
*.     : *  ...* *:*****:***.*  *****::. ** :** *:*   ***:*:

SP|P02789|TRFE_CHICK MDDFELLCTDGRRANVMDYRECNLAEVPTHAVVVRPEKANKIRDLLERQEKRFGVNGSEK 641
SP|P02788|TRFL_HUMAN LADFALLCLDGKRKPVTEARSCHLAMAPNHAVVSRMDKVERLKQVLLHQQAKFGRNGSDC 646
SP|P02787|TRFE_HUMAN EKDYELLCLDGTRKPVEEYANCHLARAPNHAVVTRKDKEACVHKILRQQQHLFGSNVTDC 634
*: *** ** *  * :  .*.** .*.**** * :*   ::.:* :*:  ** * ::

SP|P02789|TRFE_CHICK -SKFMMFESQNKDLLFKDLTKCLFKVREGTTYKEFLGDKFYTVISSLKTCNPSDILQMCS 700
SP|P02788|TRFL_HUMAN PDKFCLFQSETKNLLFNDNTECLARLHGKTTYEKYLGPQYVAGITNLKKCSTSPLLEACE 706
SP|P02787|TRFE_HUMAN SGNFCLFRSETKDLLFRDDTVCLAKLHDRNTYEKYLGEEYVKAVGNLRKCSTSSLLEACT 694
:* :*.*:.*:***.* * ** :::  .**::** ::   : .*:.*. * :*: *

SP|P02789|TRFE_CHICK FLEGK 705
SP|P02788|TRFL_HUMAN FLRK- 710
SP|P02787|TRFE_HUMAN FRRP- 698
```

Figure 2.1: Sequence alignment of chicken Ovt (P02789), Human lactoferrin (P02788) and Human serotransferrin (P02787) using CLUSTAL O (1.2.1) multiple sequence alignment (Uniprot.org).

Ovotransferrin has immunomodulatory effects, inducing respiratory burst activity and degranulation in heterophils and macrophages as well as stimulating the release of IL-6 a proinflammatory cytokine from these cells. Ovotransferrin also has an anti-inflammatory role through its inhibition of IL-1 and TNF-α *(*Xie *et al.*, 2002c). Ovotransferrin facilitates tissue remodelling and angiogenesis, aiding post inflammatory repair of tissues (Xie *et al.*, 2002c).

2.1.3.2 Ovotransferrin and the Acute Phase Response in chickens

Chickens infected with a variety of pathogens, as well as immunostimulatory chemicals, have shown to have an increase in a 65kDa serum protein that Xie *et al.*, (2002a) identified as Ovt. With reference to Table 2.1 and Figure 2.2 the majority of studies have confirmed that Ovt increases in response to a variety of experimentally induced infections and inflammatory processes.

Infectious agent	Reference
Viral	
Infectious bursal disease virus	Xie et al., 2002b
Infectious bronchitis virus	Xie *et al.*, 2002b
Respiratory enteric orphan virus	Xie *et al.*, 2002b
Fowl Poxvirus	Xie *et al.*, 2002b
Infectious laryngotracheitis virus	Xie *et al.*, 2002b
Low pathogenic avian influenza	Sylte and Suarez, 2012
Bacterial	
Escherichia coli (LPS)	Xie *et al.*, 2002b
	Rath, *et al.*, 2009
	Hallquist and Klasing, 1994
Salmonella ser Gallinarum	Garcia *et al.*, 2009
Parasite	
Eimeria tenella	Rath *et al.*, 2009
Eimeria maxima	Rath *et al.*, 2009
Immune mediated	
Autoimmune vitiligo	Rath *et al.*, 2009
Metabolic	
Tibial dyschondroplasia separation (spontaneous) (6 weeks age)	Rath *et al.*, 2009
	Durairaj *et al.*, 2009
Pulmonary hypertension (ascites) (Induced) (6 weeks age)	Rath *et al.*, 2009
Femoral head (6 weeks)	Durairaj *et al.*, 2009

Table 2.1: Infectious, immune mediated and metabolic diseases in which Ovt has been measured in chickens.

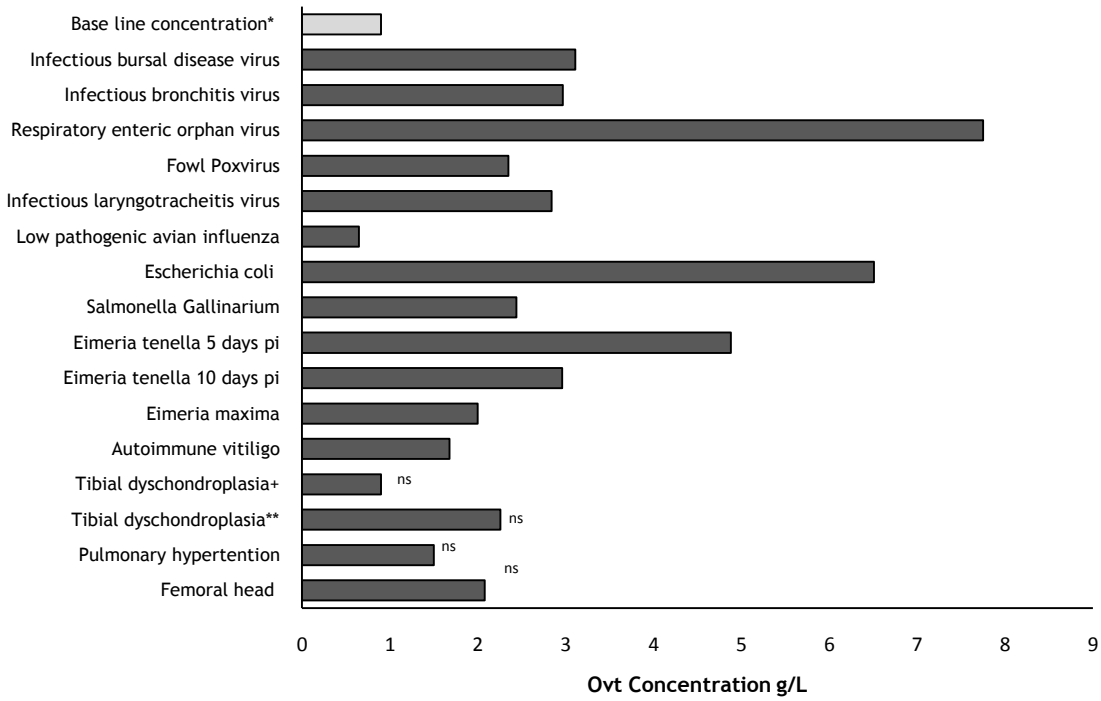


Figure 2.2: Reported serum Ovt concentrations in experimentally induced and naturally occurring disease and vaccination (Table 2.1). * Base line concentration of Ovt Xie, *et al.*, (2002), ** Durairaj *et al.,* 2009, +Rath *et al.,* 2009, *ns* not significantly different to respective controls. For the viral challenges Ovt was measured 7 days post infection (pi) (Xie *et al*., 2002) except for LPAI which was measured up to 96 hours pi (Sylte & Suarez, 2012). For *E.coli* and *S. Gallinarium* Ovt was measured 7 and 5 day pi respectfully (Xie *et al*., 2002; Garcia *et al*., 2009). *Eimeria maxima* results were obtained 7 days pi and the Autoimmune vitiligo (an autoimmune disease causes discoloured feathers, birds were 13 weeks of age when sampled (Rath *et al*., 2009). The TD and femoral head separation disorder were naturally occurring, diagnosed at necroscopy at 6 weeks of age (Durairaj *et al*., 2009; Rath *et al*., 2009).

Lipopolysaccharides (LPS) are cell wall components of Gram negative bacteria that cause inflammation and sickness and are known to induce an especially strong APR with resultant changes in APP (Xie *et al*., 2000). *Escherichia (E.) coli* LPS has been used to demonstrate that Ovt is a positive APP in chickens in a number of studies. Xie *et al*., (2000) showed that the administration of LPS from *Salmonella (S.) typhimurium* to broiler chickens resulted in significant elevations in IL-6 and a serum band shown to be Ovt. Garcia *et al*., (2009) used an oral infection route, infecting birds with *Salmonella* ser Gallinarum *per os*, as oppose to intra peritoneum and found conversely that Ovt responded negatively, decreasing in response to infection.

Xie, *et al*., (2002b) quantified the serum Ovt levels in response to a number of viruses as well as *E. coli*. The highest Ovt concentration was detected in a viral

infection. The respiratory enteric orphan virus increased serum Ovt concentration 8.6 fold, while other viruses elevated serum Ovt concentration 2.6 – 3.5 fold. Investigating the response of APPs to low pathogenic avian influenza (LPAI) vaccination and infection, Sylte and Suarez (2012) measured Ovt alongside AGP and found that chickens receiving a LPAI vaccine had significantly lower serum Ovt at 48 and 96 hours post infection than those chickens that did not receive the vaccine.

Rath, *et al.*, (2009) compared changes in the serum Ovt concentrations due to infectious diseases, metabolic diseases and autoimmune disease. In keeping with Xie *et al.*, (2002b) the infectious diseases showed the biggest increases in Ovt, with *E. coli* and protozoal pathogens *E. maxima* and *E. tenella*, causing the greatest increases in Ovt. The autoimmune disease, autoimmune vitiligo, which is an auto-inflammatory disease that results in loss of melanocytes and resulting discolouration of the feathers, resulted in birds with increased in serum concentrations of Ovt. Birds with the metabolic diseases: tibial dyschondroplasia, pulmonary hypertension syndrome and femoral head separation disorders did not show elevations in serum Ovt. It was postulated from these results that as well as inflammation, infection or tissue injury was also needed to evoke a rise in OVT (Rath, *et al.*, 2009). It appears that increases in Ovt are only evident with pathogenic challenge and the increases seen in birds with the autoimmune disorder was most likely due to the pathologically high levels of proinflammatory cytokines that are stimulatory to Ovt synthesis. Changes in the serum Ovt concentration over time have found that 6 hours post croton oil injection, serum concentrations dropped slightly, then continued to show a steady increase peaking 72 hours post injection before reducing to pre-injection concentrations 240hours post injection (Xie *et al.*, 2002b).

2.1.3.3 Quantification of Ovotransferrin in chickens

The most widely cited method to quantify Ovt in chicken serum is by immunoassay. Xie, *et al.*, (2002b) describes a competitive ELISA method utilising rabbit α-chicken serum transferrin antibody (Accurate Chemicals) and biotinylated egg Ovt (Conalbumin Sigma C0755). Rath *et al.*, (2009) describes a similar immunoassay, without the biotinylation step. A commercial immunoassay

ELISA kit is available (ICL Inc. Portland Oregan USA) and has been used with success (Roy *et al.*, 2014).

Other methods to quantify Ovt have utilised gel based methods running serum samples on SDS PAGE and using densitometric scanning to obtain a concentration (Xie *et al.*, 2002a, Garcia *et al.*, 2009). A method for measuring Ovt in birds has been adapted from Yamanishi, *et al.*, (2002) by Horrocks, *et al.*, (2011) and omits the need for an antibody and instead measures the total iron binding capacity (TIBC) in serum. Using three reactions steps, this method firstly involves saturating serum transferrin with ferric iron under alkaline conditions, before reducing unbound iron with absorbic acid and eliminating it by adding a chromogenic reagent that forms a complex with ferrozine. Finally the ferric iron is dissociated from transferrin and the formation of ferrozine-Fe^{2+} complex which is proportional to the TIBC under acidic conditions. The increase in absorbance is measured at 570 nm (primary wavelength) and 660 nm (reference wavelength) (Yamanishi, *et al.*, 2002).

2.1.4 Investigating acute phase proteins in broiler chickens

To investigate and understand the behaviour of APPs in broiler chickens three studies were utilised (Table 2.2). The first examined the APPs Cp and PIT54 in healthy and culled broilers from a commercial farm. The second investigated the effect of gait score on the APPs Cp, PIT54 and Ovt. This study also looks at the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on the same APPs. The third study compared the serum from birds experimentally infected with *Eimeria tenella* (*E. tenella*) and *Escherichia coli* (*E. coli*) to determine whether the APPs Cp and Ovt differed between groups. Proteomic investigations to examine differentially expressed protein bands was also undertaken.

Study 1	Acute phase proteins in culled and healthy broilers
Study 2	Effect of Gait score and NSAIDs on Acute phase proteins in broiler chickens
Study 3	Coccidiosis and Acute phase proteins

Table 2.2: Three studies on which APPs were measured.

2.1.4.1 Acute phase proteins in culled and healthy broilers

All of the published data on chicken APPs has described specific and highly controlled experimental infections and challenges. There is no published information regarding APP concentrations from chickens on poultry units. Sick birds on a commercial unit may either die or be culled by stock personal if showing signs of ill health such as collapse, lameness and stunting. By obtaining serum from culled birds together with samples from healthy birds from the same unit the APPs can be measured to see if the non-specific diseases that result in a bird being culled result in changes in APP concentrations. The differences between a bird housed with thousands of other birds in a commercial environment compared to a small replicate pen in a scientific study are vast and this study may also indicate whether this affects base concentrations of APPs in healthy birds.

2.1.4.2 Effect of gait score and NSAIDs on acute phase proteins in broiler chickens

Gait abnormalities in broiler chickens and the diseases and disorders that cause them are major issues for the poultry industry affecting both productivity and welfare. Investigating alternative methods to identify, prevent and control the underlying causes of gait abnormalities in broilers would be welcomed. There has been no previous attempt to characterise the APP changes that occur as a result of gait abnormalities in broilers. The Bristol six point Gait Score (GS) system (Kestin *et al.*, 1992) is a method of assessing walking style of individual birds and allocating a score of GS 0 (no detectable walking abnormality) through to GS 5 (unable to stand). This method has been widely employed in welfare studies to assess the walking ability of broilers. This is a qualitative method of assessing gait and determining the APP concentrations would determine whether the cause of a poor walking gait is purely mechanical or whether there are inflammatory factors associated with the cause of the poor walking gait. In this investigation, conducted by Dr. Vicky Sandilands at the Scottish Rural College (SRUC), birds were selected from commercial farms on the basis of having a gait score of 1 (GS1), a slight uneven gait typical of normal broiler walking, or GS3, an obvious gait defect affecting ability to move about (Kestin *et al.,* 1992).

Acute phase protein studies into specific causes of lameness in chickens are so far limited to Durairaj, *et al.,* (2009) who measured Ovt in broilers with femoral

head separation and Rath *et al.,* (2009) who also measured Ovt in chickens with tibial dyschondroplasia. Both of these studies found no significant elevations in Ovt in birds affected with these diseases, possibly because both of these diseases are metabolic diseases and in these studies did not evoke sufficient tissue damage and inflammation to cause an acute phase response. Looking to other veterinary species, lame cattle arriving at a clinic were found to have significantly elevated APPs (fibrinogen, haptoglobin and serum amyloid A) compared to clinically healthy cows (Jawor, *et al.*, 2008). Lame sows were found to have higher haptoglobin and C-reactive protein concentrations than non-lame controls (Oravainen, 2008). By assessing birds with generalised non-specific lameness as determined by gait score, this study would determine whether lameness in chickens also causes elevations in APPs.

Non-steroidal anti-inflammatory drugs are widely used analgesics for the treatment of inflammatory musculoskeletal diseases as well as fever, pain and other signs associated with inflammatory ailments (Peters *et al.,* 2012). Non-steroidal anti-inflammatory drugs target cyclooxygenases and subsequently inhibit the production of prostaglandins and thromboxanes, which are key mediators in the process of inflammation and thus provide a targeted approach to treating inflammation compared to their steroidal counterparts (Peters *et al.*, 2012). Meloxicam, though not licensed in birds has reported safety and efficacy in treating lameness, pain and joint inflammation in back yard chickens (Hadipour *et al.,* 2011). It was also the aim of this study to determine if the administration of NSAIDs to both GS1 and GS3 birds also affected the concentration of APPs.

There are few accounts describing APPs in relation to NSAIDs in veterinary species. Peters *et al.*, (2012) investigating the RNA expression of inflammatory biomarkers in LPS treated pigs, found that the NSAID flunixin meglumine reduced the expression of serum amyloid A-2 48 hours post stimulation with LPS. In a clinical study (Bennett *et al.*, 2013) found that osteoarthritic dogs treated with robenacoxib for 28 days had reduced concentrations of CRP in their synovial fluid, though serum concentrations of CRP were unchanged. These studies, like many cited in the human literature describe studies utilising NSAIDs in known cases of inflammatory diseases and at the therapeutic doses. It has been noted that at suprapharmacological doses NSAIDs will elicit pathology and gene

expression changes similar to LPS administration (Tugendreich, *et al.*, 2006). Studies in rat models found the NSAIDs indomethacin and ibuprofen induced changes in the gastrointestinal tract that allow bacteria and LPS to leak into systemic circulation and alter hepatic expression of inflammatory genes resulting in an APR (Tugendreich *et al.*, 2006). Determining whether meloxicam causes elevations in GS1 and GS3 birds could determine whether NSAIDs evoke a APR in this species, or whether the administration of NSAIDs causes no effect on the serum concentrations of APPs.

2.1.4.3 Coccidiosis and acute phase proteins

Coccidiosis is caused by protozoan parasites that are members of the genus Eimeria. Seven Eimeria species have been recognized to infect chickens: *Eimeria acervulina, E. maxima, E. tenella, E. brunetti, E. necatrix, E. mitis* and *E. praecox* (Vermeulen, *et al.*, 2001; Swaggerty, *et al.*, 2011). Each species is pathogenic to chickens in varying degrees, with *E. acervulina, E. maxima,* and *E. tenella* the most pathogenic and prevalent within the broiler industry (Swaggerty *et al.*, 2011).

Eimeria species infect the epithelial cells of the intestinal lining causing pathological changes varying from local destruction of the mucosal barrier and underlying tissue (often associated with some degree of inflammation resulting in endothelial lesions), to systemic effects such as blood loss, shock syndrome and even death (Vermeulen *et al.*, 2001). Each Eimeria species targets a distinct region in the intestinal tract resulting in a characteristic pathology at this region: *E. acervulina* pathology is characterised by white lesions in the upper small intestine, *E. maxima* results in petechiae in the midgut, and *E.tenella* targets the caecal pouches and results in bloody faeces (Hammond & Long, 1973). Coccidiosis is one of the most economically important diseases to affect commercial poultry production, costing the poultry industry upward of U$3 billion worldwide annually (Swaggerty *et al.*, 2011). At least 70% of that cost is a result of subclinical coccidiosis characterized by substandard flock performance, including decreased weight gain and increased feed conversion (Swaggerty *et al.*, 2011).

There is a great deal of interest therefore in control strategies as currently the only effective control strategy is the addition of anticoccidials to feed and

water. Vaccination development and intestinal and systemic immunity remain key areas in Coccidial research. The effect of Coccidial infection on APP concentration varies depending on the species involved. Georgieva *et al.,* (2010) found Cp and fibrinogen (Fb) to increase significantly in birds infected with *E. tenella,* and found that in combination with *E. coli*, *E. tenalla* significantly increased serum concentration of Cp, PIT54 and Fb. This study found the increases in Fb concentration to be highly significant (*P*=<0.001). Rath *et al.*, found Ovt to be significantly increased during *E. tenalla* infection both 5 and 10 days post infection. Ceruloplasmin was found to only be significantly higher 6 days post infection (Richards & Augustine, 1988). Looking at the response of Cp in response to *E. acervulina* infection, Richards & Augustine, (1988) found no significant increases in the serum concentration of this APP. Rath *et al.,* (2009) examined *E. maxima* and found this species to also significantly increase Ovt concentrations. Table 2.3 details the serum concentrations of these APPs in response to Eimeria challenge.

Coccidial species	APP measured	Control APP concentration g/L	Post-infection APP concentration g/L	Reference
E. tenella	Cp (8 days pi)	0.052 ± 0.01	0.098 ± 0.017*	Georgieva *et al.*, 2010
	PIT54 (8 days pi)	0.100 ± 0.02	0.124 ± 0.007	
	Fb (8 days pi)	0.99 ± 0.11	1.80 ± 0.17*	
	Ovt (5 days pi)	1.20 ± 0.16	4.88 ± 0.37*	Rath *et al.*, 2009
	Ovt (10 days pi)		2.96 ± 0.32*	
	Cp (3 days pi)	0.12 ± 0.001	0.12 ± <0.001	Richards and Augustine, 1988
	Cp (6 days pi)		0.20 ± 0.001*	
E. tenella* & *E. coli**	Cp	0.052 ± 0.01	0.14 ± 0.03*	Georgieva *et al.*, 2010
	PIT54	0.100 ± 0.02	0.150 ± 0.008*	
	Fb	0.99 ± 0.11	3.35 ± 0.43*	
E. maxima	Ovt (7 days pi)	1.12 ± 0.18	2.00 ± 0.24*	Rath *et al.*, 2009
E. acervulina	Cp (3 days pi)	0.12 ±<0.001	0.135 ± <0.001	Richards and Augustine, 1988
	Cp (6 days pi)		0.134 ±0.001	

Table 2.3: Coccidial species and APP concentrations before and after infection.

*significantly different from respective controls/pi – post infection/** Sample for APP analysis taken 8 days and 4 days post infection for *E.tenella* and *E.coli* respectfully.

2.1.5 Aims of study

The aims of this chapter were to determine the APP response in chickens in three areas of poultry health. The APPs Ovt, Cp and PIT54 were targeted for measurement owing to the availability of these assays and previous validation steps undertaken for use in chickens. The first question addressed was whether culled birds had significantly different APP expression compared to healthy birds (study 1). By measuring the APPs Cp and PIT54 the normal basal values in birds from commercial farms can be established and used for future reference. The measurement of APPs in culled chickens from commercial farms has not been reported and although a basic undertaking in principle, determining whether generalised ill-health results in an elevation in APP concentrations would be advantageous to future applications of APP measurement in chickens. By measuring APPs and undertaking proteomic investigations in birds from studies investigating lameness and NSAIDs (study 2) and Coccidiosis (study 3), it was the aim of this chapter to determine what effect these diseases have on APP expression and acute phase sera. It was hypothesised that culled birds would have higher concentrations of circulating APPs (study 1) and that because lameness may be associated with pathological conditions it was hypothesised that an increased gait score would result in higher concentrations of APPs (study 2). Previous studies focusing on APPs during Coccidial infections have found that these infections cause elevations in APPs in chickens, and so it was hypothesised that infection with *E. tenella*, particularly when associated with *E. coli* would cause significant increases in APP concentrations (study 3).

2.2 Materials and Methods

2.2.1 Acute phase protein assay validation and measurement

2.2.1.1 Reagents

Chemicals and materials used were obtained from Sigma Chem. Co. Poole, UK and Bio-Rad Laboratories Ltd Hemel Hempstead UK. Unless otherwise stated. Double distilled water was used throughout.

2.2.1.2 Bradford assay

Bovine serum albumin (BSA), used as a standard was diluted in 0.9% NaCl from 2mg/ml to 0.25mg/ml. Samples were diluted with 0.9% NaCl 1:5 , 1:10, 1:20 or 1:40 as deemed necessary, and 5µl of sample and each standard were added in triplicate to wells of a 96 well plate (Falcon # 351172). Bradford reagent (Sigma #SLBC4590V) was warmed to room temperature and 250µl was added to each well. The plate was placed on a plate shaker for 30 seconds before incubating at room temperature for 5 minutes, after which the absorbance was read at 595nm on Flurostar optical density reader (BMG LABTECH, Ortenburg Germany).

2.2.1.3 Ceruloplasmin assay

Serum Cp was measured indirectly using p-phenylenediamine (PPD) oxidase activity. Cp catalyses the oxidation of PPD to yield a purple coloured product whose rate of formation can be determined by spectrophotometry. The rate of formation of the coloured oxidation product is proportional to the concentration of serum Cp (Sunderman and Nomoto, 1970). Before applying the PPD assay to chicken serum the optimum pH was established according to (Martinez *et al*, 2007) and found to be pH 6.2. The PPD reagent was made by adding 61.5mg of p-phenylene di amine dihydrochloride (sigma P1519) to 25ml of 0.598M sodium acetate buffer pH 6.2. Porcine serum of a known Cp concentration (118mg/ml) was diluted 1:100 and serially diluted to achieve a standard curve. To each well of a 96 well plate 50µl of PPD and 15µl of serum sample or standard solution was added. This was left in the dark for 20 minutes and the read on a Flurostar optical density reader at 550nm (BMG LABTECH, Ortenburg Germany). A Pentra

biochemical auto analyser (Horiba Medical, Montpellier France) was used subsequently to measure large numbers of samples using the same method. Coefficient of Variants (CVs) for the Cp assay were calculated and are detailed in Table 2.4.

Intra-assay CV	Mean Concentration (n=3)	SD	CV
Sample 127 (low)	1.85	0.27	14.62
Sample 221 (high)	3.34	0.34	10.14
	Intra-assay CV		**12.37**
Inter-assay CV	Sample run	QC-low	QC-high
	16.12.10	1.53	-
	29.09.11	1.41	2.58
	23.11.11	1.38	2.55
	17.12.13	0.5	1.96
	21.02.12	1.17	2.35
	27.06.12	1.31	2.39
	Mean	1.22	2.37
	SD	0.37	0.25
	CV	30.49	10.46
	Inter-assay CV		**20.47**

Table 2.4: Coefficient of Variants (CVs) for the Cp assay. Intra-assay CVs were calculated from the SD of the mean (n=3) to determine the within plate variation. Two samples were used: a high and low reading sample. The inter-assay CV was calculated from running two samples (a high and low) over six assay runs. The values of which were then used to calculate a CV to determine repeatability.

2.2.1.4 PIT 54 assay

Commercial assay methods for measuring haptoglobin, the haemoglobin binding protein in mammals, has been found to be effective for chicken PIT54 (Georgieva *et al*. 2010). The peroxidise activity of haptoglobin (or PIT54 when measured in chickens) is directly proportional to the concentration of the protein (Eckersall et al., 1999). A Pentra biochemical auto analyser (Horiba Medical, Montpellier France) was used to measure this activity and calculate the concentration of PIT54. Table 2.5 details the calculated CVs for this assay.

Intra and Inter-assay Coefficient of Variants		
Intra assay	High	17%
	Low	15%
Inter-assay	High	7%
	Low	7%

Table 2.5: Coefficient of Variants (CVs) for the haptoglobin/PIT54 assay. Intra-assay CVs were calculated from the SD of the mean (n=12) to determine the within plate variation. Two samples were used: a high and low reading sample. The inter-assay CV was calculated from running two samples (a high and low) over twelve assay runs. The values of which were then used to calculate a CV to determine repeatability.

2.2.1.5 Ovotransferrin Competitive ELISA

An Ovt assay detailed by Narayan Rath (Rath *et al*. 2009; 2010, *pers comm*.) was modified and used to determine the concentration of Ovt. A 96 well plate was coated with chicken Ovt (Conalbumin Sigma C0755) at a concentration of 1µg/ml in 0.2M Sodium hydrogen carbonate and stored at 4°C overnight. Wells were washed with Tris buffered saline (18mM Tris . 123.2mM NaCl) (TBS) and blocked with 0.5% (v/v) fish gelatin (Sigma G7765) for 1 – 2 hours and removed before the addition of the test solutions.

Ovotransferrin standards were made by serially diluting 32µg of OVT in 1 ml TBS to 0.125µg/ml. Rabbit anti-chicken transferrin (i.e. Ovt) antibody (Accurate Chemical AI-AG 8240) was diluted to a concentration of 0.2µg/ml in tris-buffered saline (TBS). Serum was diluted 1:20 in TBS. To wells 50 µl of diluted standards or samples were added in duplicate and to this 50 µl anti-chicken transferrin antibody was added, mixed and incubated by rocking for 2 hr. The wells were washed successively 4-5 times using TBS containing 0.05% tween-20 (TBS-T). To each well 100µl of 0.02% goat anti-rabbit IgG-HRP (abcam ab6721) was added. After 1 hour incubation by constant shaking, each well was aspirated and washed 3-4 times with TBS-T. To each well 100µl of IMB peroxidase substrate (KPL 50-76-00) was added and incubated for 4 – 5 minutes at room temperature as the wells turned blue. The reaction was halted with the addition of 100µl 1M HCl. Absorbance was measured at 450nm on a FLUOstar absorbance microplate

reader and analysed using associated MARS data analysis software (both BMG LABTECH, Ortenburg Germany) to determine OVT concentrations of samples.

Coefficient of Variants (CVs) for the Ovt competitive ELISA were calculated using 108 samples across 8 plates and 4 quality control samples and the results detailed in Table 2.6.

Coefficient of Variants		**SD**	**CV**
Intra-assay CV		10.71	**14.91%**
	Sample		
Inter-assay CV	20	0.82	36.35
	37	0.81	49.14
	234	0.64	28.33
	33	0.17	14.43
	Average		**32.06%**

Table 2.6: Coefficient of Variants (CVs) for Ovt competitive ELISA. Intra-assay CVs were calculated from the SD of the mean of 108 samples measured in triplicate to determine the within plate variation. The inter-assay CV was calculated from running four samples over eight plates. The values of which were then used to calculate a CV to determine repeatability.

2.2.2 Proteomics

2.2.2.1 One and two dimensional electrophoresis SDS PAGE (1DE/2DE)

To compare the protein profiles on one-dimension SDS PAGE (1DE) each serum sample was vortexed and diluted 1:10 with dH_2O, 2µl of XT reducing buffer (BioRad #161-0788) and 10µl of XT sample buffer (BioRad #161-0791) was added, heated at 95°C for 4 minutes and pipetted into the well of a BioRad Criterion XT Bis-Tris precast 18 well gel (BioRad #345-0124). A molecular weight marker was added to one well and the gel was ran at 200V for approximately 25 minutes (just before the protein front reached the edge of the gel) in MOPS running buffer (BioRad #161-0788). The gel was removed from the cassette and stained in Coomassie blue G dye (Sigma-Aldrich, Dorset, UK) for an hour and de-stained in 10% (w/v) acetic acid in 25% (v/v) methanol overnight .

For two-dimensional (2DE) separation 200ug of protein at a concentration of 1µg/µl was used on each gel. 200µg of protein was diluted in rehydration buffer (8M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® BioRad 163-2106) and loaded onto 11cm 3-10 pH non-linear IPG strip (BioRad ReadyStripTM) covered in 500µl of mineral oil and focused overnight with a combined rehydration - focusing step over 17 h totalling of 35,000 V-hr (PROTEAN IEF cell protocol, BioRad). For the second dimension IPG strips were firstly reduced for 10 minutes with 64.83 mM Dithiothreitol (DTT) dissolved in an equilibration buffer (0.374M Tris . 2.4M Urea . 13.68mM Glycerol . 69.36mM SDS) then 67.58 mM of acylated Iodoacetamide dissolved in that same equilibration buffer for 10 minutes, after which the strip was inserted into the well of a IPG+1 TGX precast 4-12% acrylamide gel (BioRad #567-1081). The gel was run for 50 minutes in MOPS buffer at 200V. Gels were stained in Coomassie for an hour and left to de-stain in acetic acid and methanol overnight for 24 hours.

2.2.2.2 Western Blotting

The gel (1DE or 2DE) for Western blotting was removed from the cassette and placed directly onto nitrocellulose paper (NCP) (Bio-Rad 162-0112) cut to size. Using a Criterion blotter (Bio-Rad 170-4070) the gel and NCP was placed between blotting paper and foam pads on both sides and sealed within a cassette holder and placed into the tank with two ice packs. Transfer buffer (25.012 mM Tris . 191.82 mM Glycine . Methanol 20 % (v/v)) was added to the tank and the transfer made at 70V over 1 hour. The NCP was blocked overnight with 1% (w/v) non-fat milk protein in TBS. The following day the NCP paper was washed three times with TBS with 0.1% tween (TBS-T). The primary antibody was diluted in TBS-T and poured over the NCP paper and placed on the rocker for 1 hour. The NCP was washed three times with TBS-T before the addition of the secondary antibody conjugated to HRP and diluted with TBS-T and placed on the rocker for a further hour. The NCP was washed three times with TBS-T and developed with the addition of Opti-4-CN (BioRad #170-8235). The NCP was finally washed with dH_2O to halt the development.

2.2.2.3 Identification of proteins using mass spectrometry

2.2.2.3.1 In gel digestion

Protein bands and spots of interest were excised from the gel, diced if necessary and placed into Eppendorf tubes and washed in 500µl of 100mM ammonium bicarbonate for 30 minutes. This wash was poured away and the gel pieces washed twice in 500µl of 50% acetonitrile in 100mM ammonium bicarbonate for 30 minutes to de-stain the gel pieces. The gel pieces were finally washed in 50µl of 50% acetonitrile to shrink the gel pieces for 10 minutes, after which the solvent was removed and the gel pieces air dried completely in a vacuum centrifuge for 2 hours. In gel digestion was performed with sequencing grade trypsin (Promega, WI USA, V511A) which was re-suspended in 20µl of 25mM ammonium bicarbonate and added to the dried gel pieces. Once the trypsin has absorbed and the gel pieces fully rehydrated a further 20µl of 25mM ammonium bicarbonate was added over the gel pieces. The protein was allowed to digest overnight at 37°C. The following day the Eppendorf's were pulse centrifuged to pellet the gel pieces and the liquid transferred to a 96 well plate. To the remaining gel pieces, 20µl of 5% formic acid was added and allowed to incubate for 20 minutes on the shaker after which 40µl of acetonitrile was added and the incubation continued for a further 20 minutes. The gel pieces were pelleted and the liquid added to the 96 well. The combined extracts on the plate were dried down completely in a vacuum centrifuge.

2.2.2.3.2 Protein identification

Protein were identified using nanoflow HPLC electrospray tandem mass spectrometry (nLC-ESI-MS/MS), in Glasgow Polyomics, in collaboration with Dr. Richard Burchmore. Peptides were solubilised in 2 % acetonitrile with 0.1 % trifluroacetic acid and fractionated on a nanoflow uHPLC system (Thermo RSLCnano) before online analysis by electrospray ionisation (ESI) mass spectrometry on an Amazon ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings). Peptides were desalted and concentrated for 4 min on a C18 trap column followed by an acetonitrile gradient for a total time of 45 min. A fixed solvent flow rate of 0.3 µl / min was used for the analytical column. The trap column solvent flow rate was fixed at 25 µl / min using 2 % acetonitrile with 0.1% v/v trifluroacetic acid. Mass spectrometric (MS) analysis was performed using a continuous duty cycle of survey MS scan followed by up to ten MS/MS analyses of the most abundant peptides, choosing the most intense multiply

charged ions with dynamic exclusion for 120s (Burchmore, *pers comm*. 2013). MS data was processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.4.1). Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI Genbank databases for *Gallus gallus* and bony vertebrates, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses (Burchmore, *pers comm*. 2013). Searches were made in both the *Gallus gallus* database and the bony vertebrate database, the latter being more extensive and therefore used to ensure that any proteins, not yet annotated for or identified in *G.gallus* could be putatively matched and identified.

2.2.3 Investigating acute phase proteins in broiler chickens

2.2.3.1 Study 1: Acute phase proteins in culled and healthy broilers

To establish whether the APPs Cp and PIT54 were elevated in birds culled due to ill health and poor performance, two groups of chickens were sampled from a commercial broiler farm in Scotland. Blood samples (serum) were taken immediately post mortem from two groups of chickens: control chickens (n=20) with no obvious signs of disease and culled chickens (n=18), birds removed from the flock by stock personal due to poor growth rate, as evidenced by stunted growth, or lameness. These birds were culled and blood sample taken immediately post-mortem intracardially.

2.2.3.2 Study 2: The effect of gait abnormality and NSAID on plasma acute phase protein concentration and plasma proteome.

Bloods were received from collaborators Dr. V. Sandilands and Professor N. Sparks at Scotland's Rural College (SRUC) from their study conducted to investigate the effects of NSAIDs on the gait score of commercially reared broilers (approved by the SRUC ethics committee). At the SRUC, 132 male Ross and Cobb broiler chickens aged 35-36 days were selected from a number of commercial farms from the same company on the basis of having a gait score of 1 (GS1), a slight uneven gait typical of normal broiler walking, or GS3, an obvious gait defect affecting ability to move about (Kestin *et al.,* 1992). Birds

were weighed and injected with either saline (S), 2mg/kg meloxicam (M2) or 5mg/kg meloxicam (M5). Blood was recovered from the birds when culled 6, 30, 54 or 78 hours after meloxicam injection and the serum concentrations of the APPs ceruloplasmin, PIT54 and ovotransferrin were determined. For analysis of the acute phase reactive plasma (APRP) proteome of the GS3 birds, samples were ranked according to plasma concentrations of Cp, PIT54 and OVT and the plasma samples (n=3) with the highest across all APPs selected. Acute phase protein results from the GS1 birds were also ranked and samples with the lowest APP concentrations selected (n=3) for further proteomic investigation (non-APRP). The plasma proteomes was analysed using 2DE and spots were selected based on differential expression between the two gait groups.

2.2.3.3 Study 3: Serum Ceruloplasmin and Ovotransferrin in chickens infected with *Escherichia coli* and *Eimeria tenellla*.

Serum samples were received from Professor Teodora Georgieva, of Trakia University, Bulgaria. Samples were from three groups of chickens. In this study (approved by Trakia University Ethics committee) group 1 were challenged orally with *E. tenella* oocysts (n=5), group 2 were challenged with both oral *E. tenella* oocysts orally and *E. coli* via intraperitoneal injection (n=7) and group 3 were healthy controls (n=11). The experiment was conducted under the same conditions and applying the same methods described in Georgieva *et al*. (2010). Samples underwent SDS PAGE in a single dimension to separate the serum proteins to identify Ovt using mass spectrometry. The serum concentrations of Ovt and Cp were determined as previously described.

From the 1DE gel bands of interest were excised for identification using mass spectrometry. Bands of interest were selected by identifying bands differentially present between samples. Selected individual protein bands were cut from the gel, trypsin digested into peptides and analysed using mass spectrometry and MASCOT database searching (Glasgow Polyomics, University of Glasgow).

2.2.4 Statistics

The results for studies 1 and 3 were analysed using GraphPad Prism v5. The data was evaluated using D'Agostino-Pearson omnibus normality test. Non-parametric analysis was selected based on the results with a Kruskal-Wallis with Dunn post-

test analysis employed for all analysis except for study 1. For this study the initial analysis employs a student t-test. With assistance from Dr. Claire Walls (University of Glasgow) the results for study 2 were analysed as a general linear mixed models with the 'R' statistical program v2.12.1. (Walls *pers comm*. 2012).

2.3 Results

2.3.1 Investigating acute phase proteins in broiler chickens

2.3.1.1 Study 1: Acute phase proteins in culled and healthy broilers

The serum concentrations of the APPs Cp and PIT54 were determined from culled and healthy birds from a commercial farm. A t-test revealed a statistically significant (p=<0.05) difference in both Cp and PIT54 between serum from culled (n=18) and healthy birds (n=20) (GraphPad Prism v.5). Culled birds had increased serum concentrations of both APPs (Figure 2.3) when compared to apparently healthy birds within the same flock.

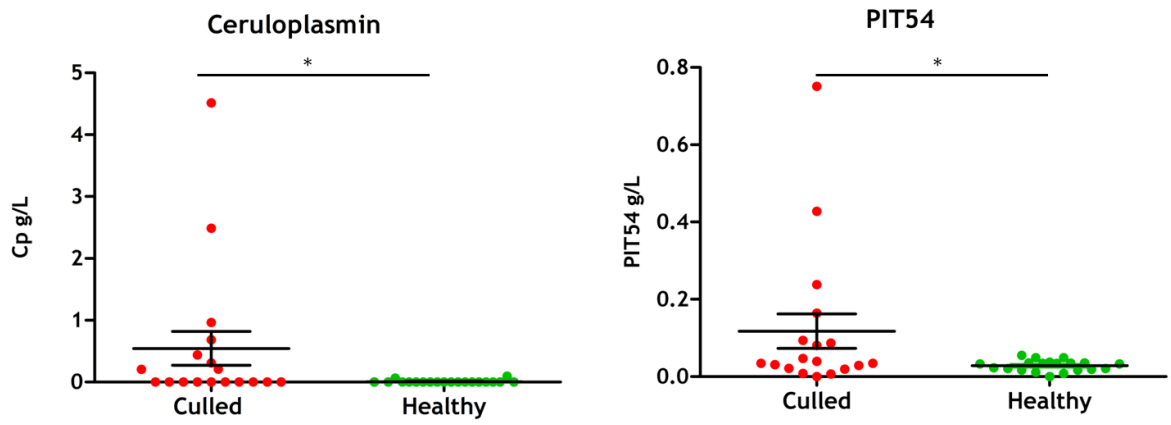


Figure 2.3 : Scatter plot of Cp and PIT54 concentrations of culled (n=18) and healthy (n=20) birds from a commercial farm, with mean (horazontal bar) and ± SEM. Data was analysed using t-test (*p<0.05) using GraphPad Prism v5.

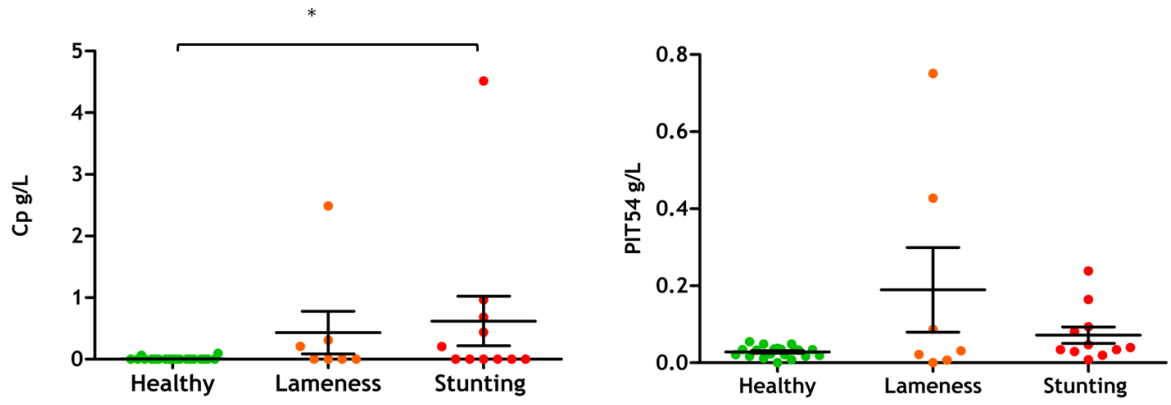


Figure 2.4: Scatter plot of Cp and PIT54 concentrations of lame (n=7), stunted (n=11) and healthy (n=20) birds from a commercial farm, with mean (horazontal bar) and ± SEM. Data was analysed using Kruskal Wallis test with Dunn post test analysis (*p<0.05) using GraphPad Prism v5.

When the birds culled were divided into those culled for stunting and those culled from lameness, a non-parametric Kruskel-Wallis test, with Dunn post test analysis was performed on the three groups. This revealed no significant difference in PIT54 concentration between the three groups. When the same test was applied to the Cp concentrations there was a significant difference between the three groups, the Dunn post test analysis revealing this to lie between the healthy and stunting. Figure 2.4 details graphically the serum concentrations of PIT54 and Cp in these three groups and Table 2.6 contains the descriptive statistics.

Group	APP	N	Min	Max	Mean	Median	SD
Healthy	PIT54	20	0	0.0550	0.0285	0.0330	0.01431
	Cp	20	0	0.0980	0.00815	0.0	0.02565
Lameness	PIT54	7	0	0.7510	0.1894	0.0310	0.2901
	Cp	7	0	2.491	0.4304	0.0	0.9174
Stunting	PIT54	11	0.0080	0.2380	0.07173	0.0400	0.07055
	Cp	11	0	4.516	0.6191	0.0	1.335

Table 2.6: Descriptive statistics for APP concentrations for culled and healthy birds. Data calculated using D'Agostino-Pearson omnibus normality test (GraphPad Prism v5).

2.3.1.2 Study 2: The effect of gait abnormality and NSAIDs on plasma acute phase protein concentration and plasma proteome.

Serum concentrations of all three APPs were significantly associated with gait, with GS3 birds having significantly higher Cp ($p \leq 0.001$), PIT54 ($p \leq 0.05$) and Ovt ($p \leq 0.05$) than GS1 birds. These results are described in Table 2.7 and Figure 2.5.

	Gate score 1			Gait score 3		
	lowest	Highest	Median	lowest	Highest	Median
Cp g/L	0.275	2.352	0.698	0.037	4.440	0.815
PIT54 g/L	0.008	0.186	0.105	0.059	0.188	0.115
Ovt g/L	0.750	3.668	2.210	0.413	11.903	2.065

Table 2.7: Cp, PIT54 and Ovt concentration ranges and medians in GS1 and GS3 birds.

Cp was significantly associated with weight with heavier birds tending to have higher Cp concentrations ($p \leq 0.05$). Birds treated with 5mg/kg of meloxicam (M5) had significantly increased Ovt concentrations, though no significant

increase was observed in birds receiving 2mg/kg of meloxicam (M2) (Figure 2.6). There were no significant associations between the APP concentrations and broiler breed or time when the meloxicam was administered.

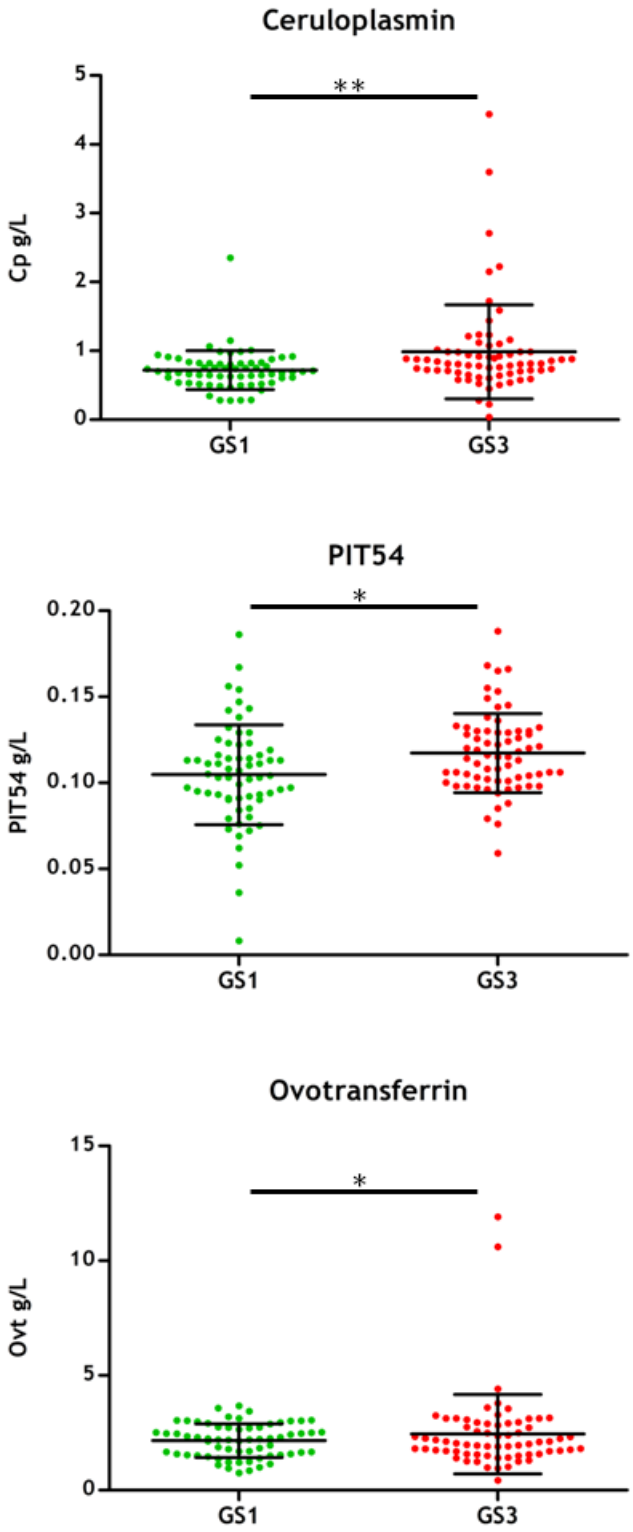


Figure 2.5: Scatter plot of Cp, PIT54 and Ovt plasma concentrations in GS1 (n=64) and GS3 (n=68) birds. Mean (horizontal bar), ± SD and significance is indicated *p≤0.05/**p≤0.001 (GLM,'R' v2.12.1).

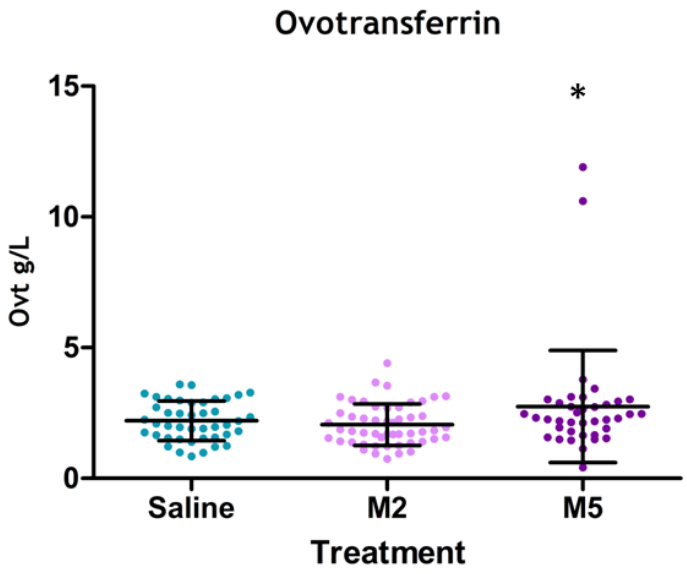
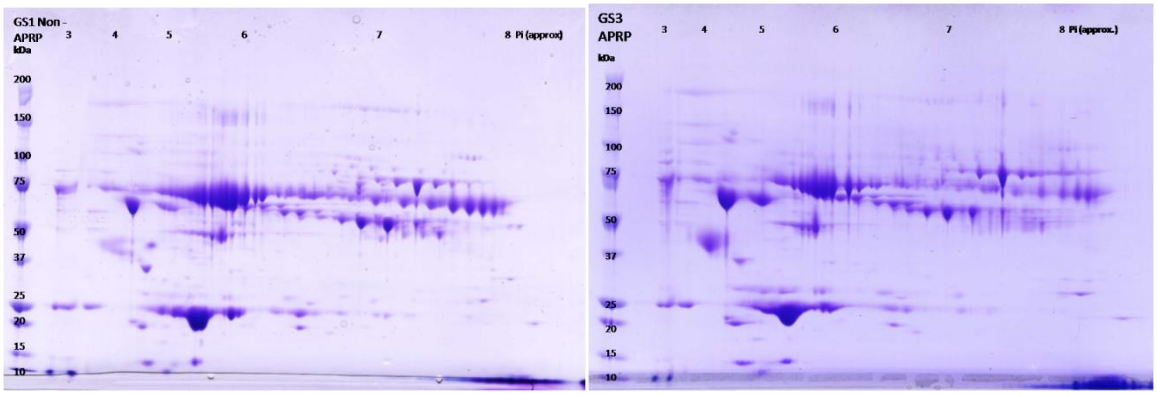


Figure 2.6: Scatter plot of Ovt plasma concentrations in saline (n=43), meloxicam at 2mg/kg (M2) (n=51) and 5 mg/kg (M5) (n=38) treated birds. Mean (horizontal bar), ± SD and significance is indicated *p≤0.05 (GLM,'R' v2.12.1).

APP concentration was used to select plasma from the most acute phase reactive GS3 birds and compared to non-APRP from GS1 birds. Differences were found in a number of regions on the APRP and non-APRP gels, with many spots differing in presence and intensity. The 3 non-APRP samples gave consistent plasma proteomes (Figure 2.7 a).There was more variation in the proteomes of the 3 APRP samples, with additional spots appearing and apparent differences in the intensity of spots (Figure 2.7b).



APRP plasma Non-APRP

Figure 2.7 a: Non-APRP and acute phase plasma

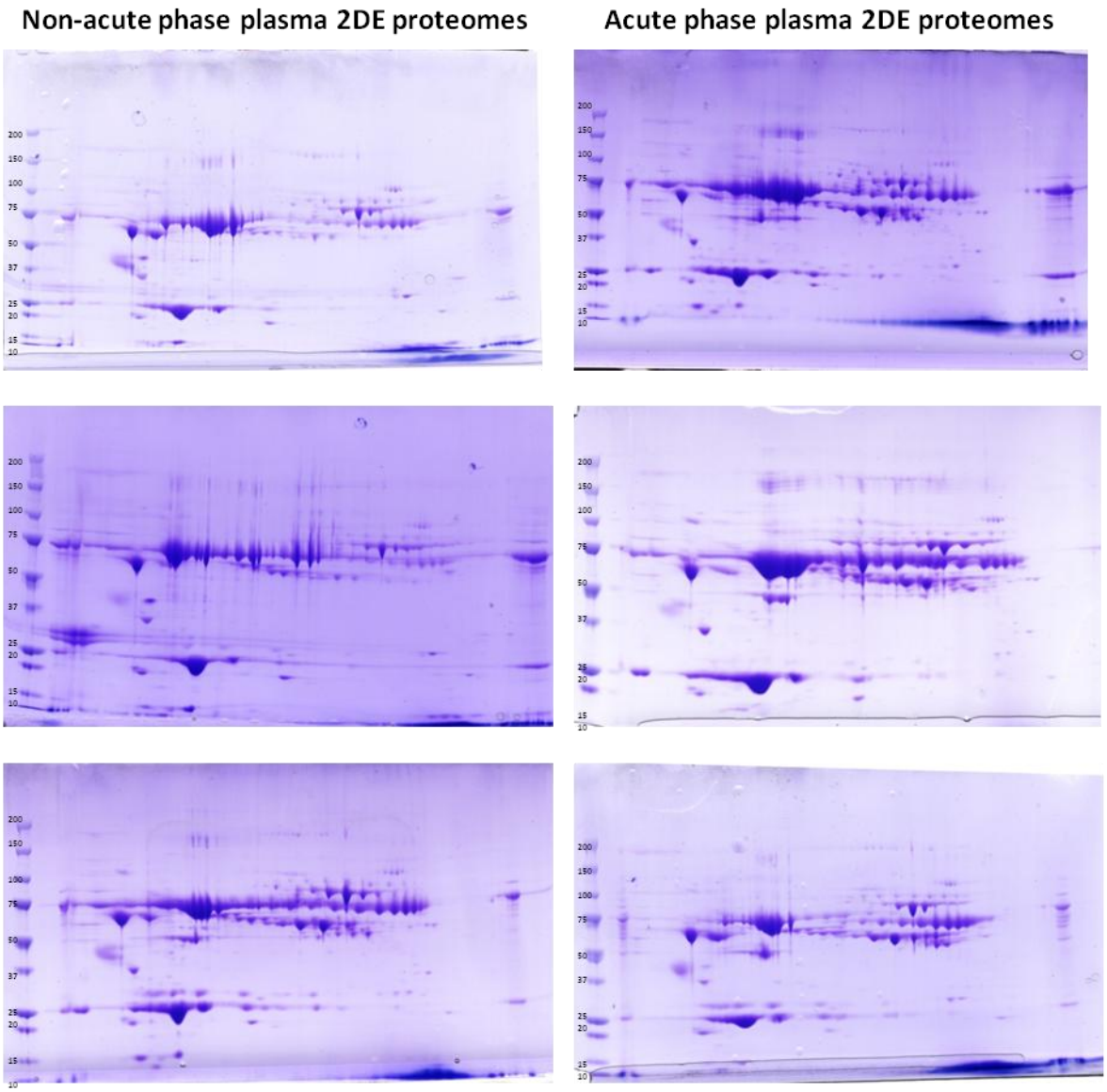


Figure 2.7 b: Six 2DE gels from APRP (left) and non-APRP (right).

Figure 2.7: a) 2DE gels comparing non-acute phase reactive plasma (Non-APRP) (a) and acute phase reactive plasma (APRP). b) Comparison of three APRP and three non-APRP gels. Separation was achieved over pi 3-10 and molecular weights of 10-200kDa. An IPG+1 TGX precast midi 4-12% acrylamide gel (BioRad#567-1081). Protein is stained with Coomassie blue G dye (Sigma Aldrich).

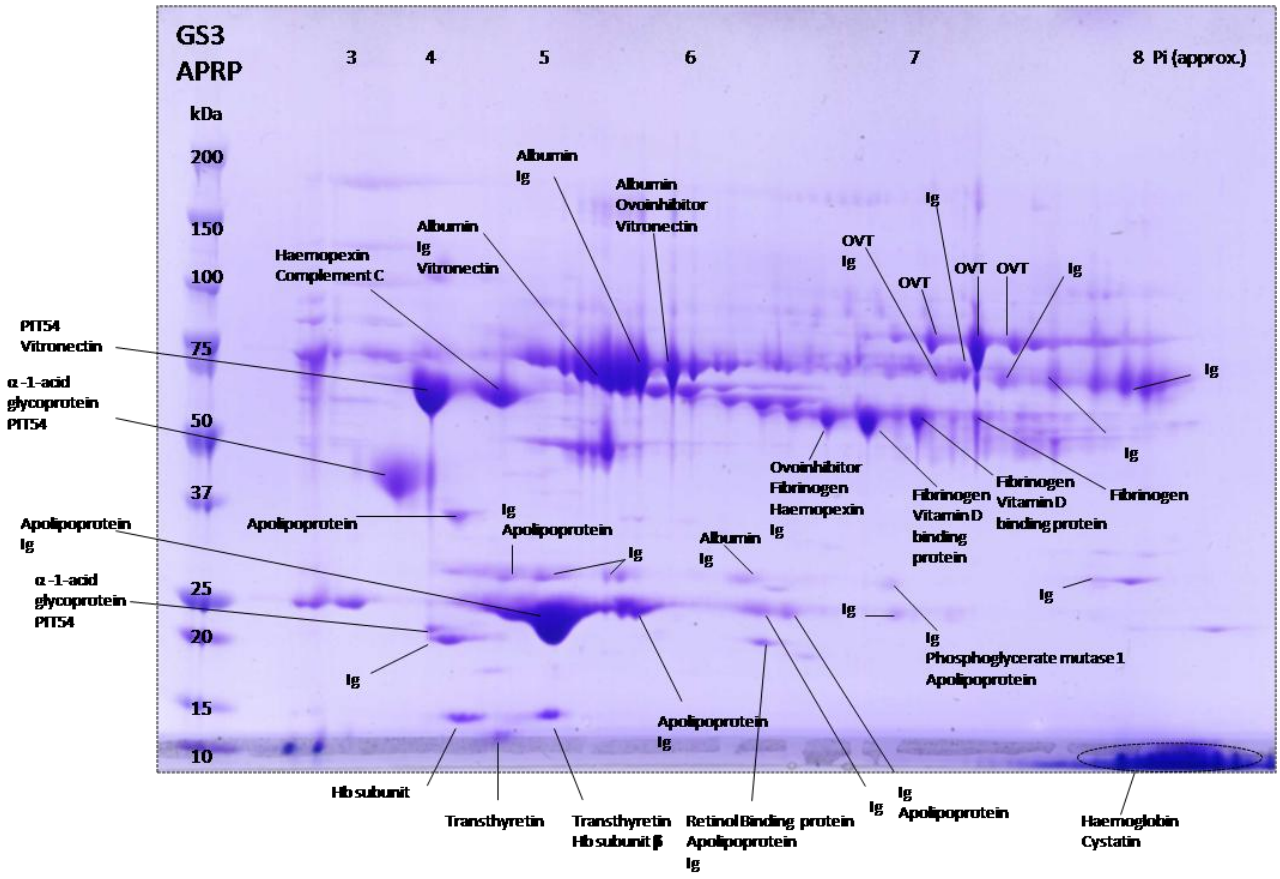


Figure 2.8: Summary of proteins found in GS3 broiler, identified using mass spectrometry. Separation was achieved over pi 3-10 and molecular weights of 10-200kDa. An IPG+1 TGX precast midi 4-12% acrylamide gel (BioRad#567-1081). Protein is stained with Coomassie blue G dye (Sigma Aldrich). Spots were excised from the gel trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates.

Mass spectrometry and peptide fingerprinting identified proteins labelled in Figure 2.8. Established APPs Ovt and PIT54 were identified, however Cp was not identified on the 2DE gel. Other APPs identified included haemopexin (Hpx), AGP and fibrinogen (Fb). In contrasting the plasma proteomes of APRP from the GS3 birds to the non-APRP of the GS1 birds, the areas corresponding to immunoglobulin (Ig) have increased while the Ovt spots are of a higher intensity in the APRP from the GS3 birds. The AGP spot was well defined in the APRP gels and was either faint or completely absent in the non-APRP gels. A full and comprehensive protein identification list for the gel in Figure 2.8 is contained in

Appendix 2.5, and corresponds to the same gel with the spots numbered (Figure 2.8).

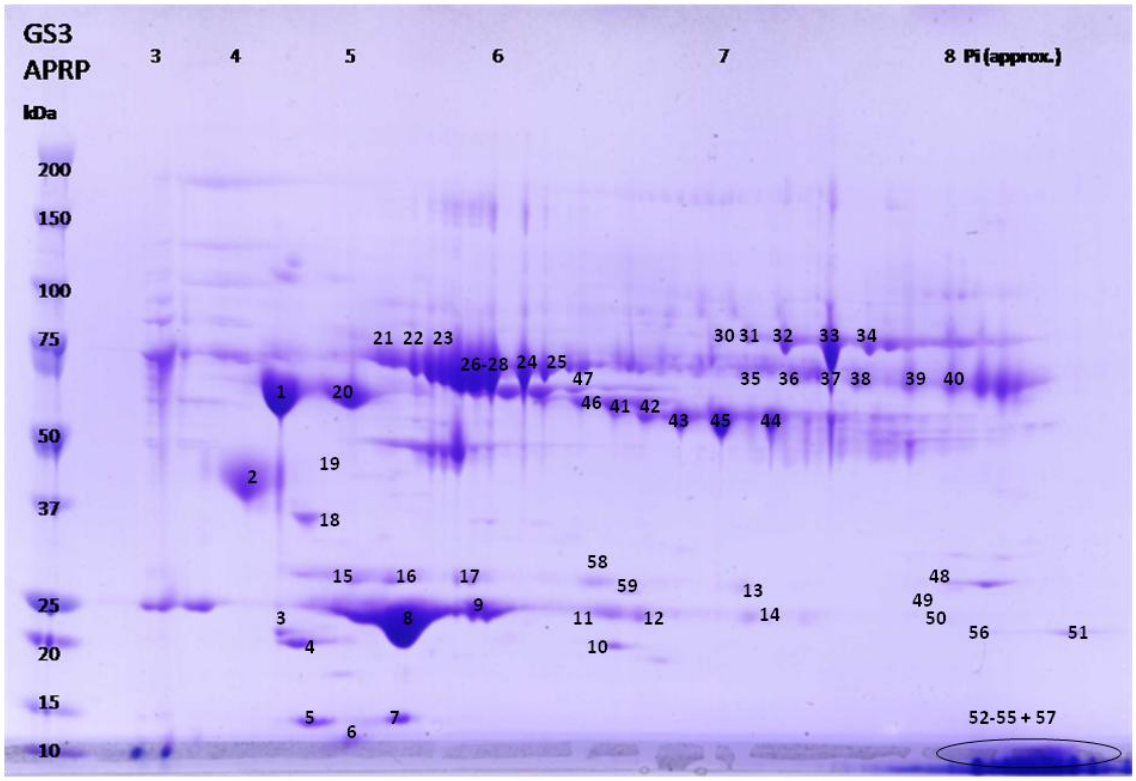


Figure 2.9: Gait gel with numbered labels. Separation was achieved over pi 3-10 and molecular weights of 10-200kDa. An IPG+1 TGX precast midi 4-12% acrylamide gel (BioRad#567-1081). Protein is stained with Coomassie blue G dye (Sigma Aldrich). A full detailed list of results is available in Appendix 2.5

2.3.1.3 Study 3: Serum Ovotransferrin and Ceruloplasmin in chickens infected with *Escherichia coli* and *Eimeria tenellla*

A non-parametric Kruskal – Wallis test revealed the serum concentration of Ovt to be significantly affected by *E. tenella* and *E. coli* infection, with Dunn post-hoc analysis indicating that the birds infected with both pathogens to have significantly higher Ovt than the control birds (Figure 2.10). Birds infected with *E. tenella* alone did not have significantly higher Ovt than the control birds. A highly significant association ($p \leq 0.0001$) was found between infection group and serum Cp concentration, with Dunn post-hoc analysis revealing both the combination infection of *E. tenella* and *E. coli* and *E. tenella* alone groups to have significantly higher Cp than the control group with p values of ≤ 0.01 and ≤ 0.05 respectfully.

From a 1DE gel loaded with a control sample, three serum samples from individual birds infected with *E. tenella* and four from birds infected with both *E. tenella* and *E. coli;* bands showing differential intensity in Coomassie staining were selected, excised and identified using mass spectrometry (Figure 2.11/2.12). The proteins identified are listed in Table 2.8. Ovotransferrin was identified with a molecular weight of 77.7 kDa. This band was present in all infected samples and no band was identified in the control sample at this molecular weight.

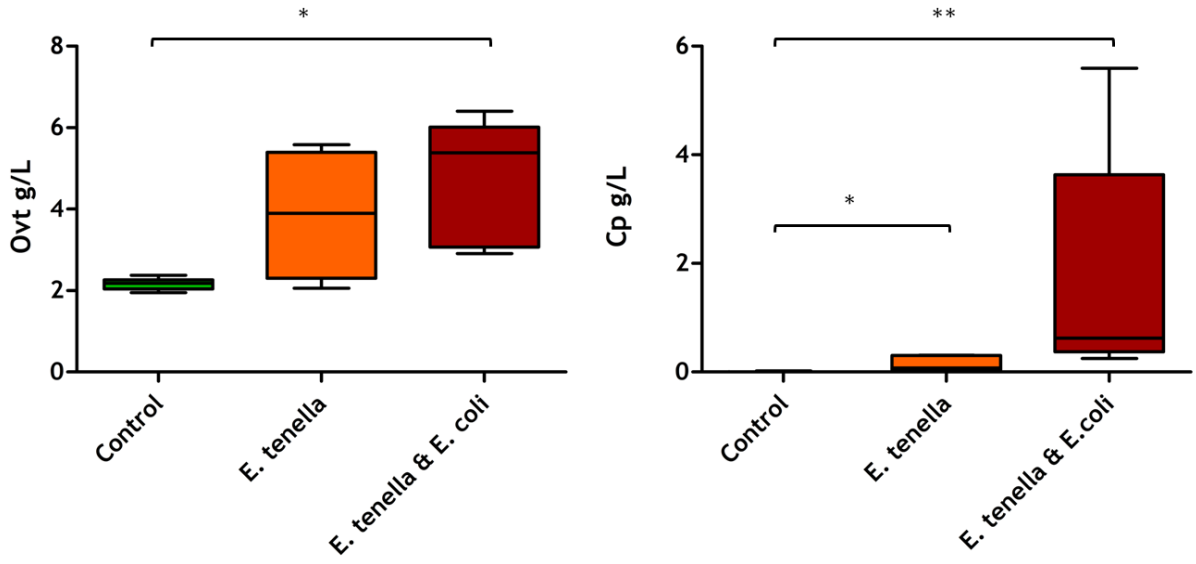


Figure 2.10: Boxplot graph describing serum Ovt and Cp from birds infected with *E.tenella* (n=5), *E.tenalla* and *E.coli* (n=7) and a control group (n=11). Data presented in median with 25 to 75 percentile range. Statistical significance (Kruskal-Wallis and Dunn post-test analysis) is reported: *p<0.05, **p<0.01 (Graphpad Prism v5).

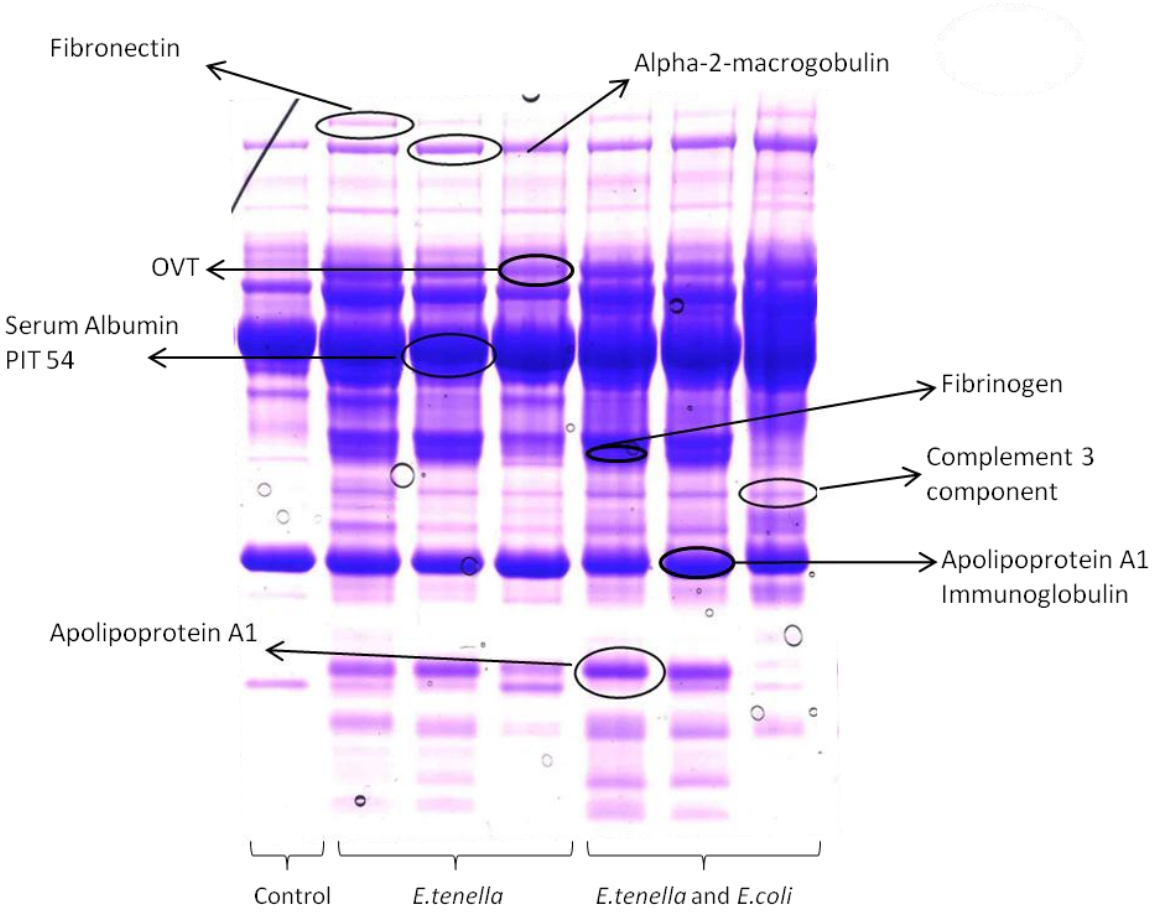


Figure 2.11: 1DE gel with serum from *E. tenella* and *E. tenella* and *E. coli* infected birds. Separation was achieved using a 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124). Protein is stained with Coomassie blue G dye (Sigma Aldrich).

	Name	Protein Identification		Score	Theoretical		Matched	Sequence	Coverage %
		UniProt Accession	NCBI Identifier		Mass Da	PI			
1	fibronectin [Gallus gallus]	P11722	gi|311213923	472	276669	5.43	14(3)	14(3)	6
2	Alpha-2-macroglobulin precursor(Alpha-2-M) [Gallus gallus]*	P98157	gi|118083282	698	164165		15(7)	15(7)	**
	Ovotransferrin	P02789	gi|1351295	70	79551	6.85	2(0)	2(0)	2
3	Ovotransferrin	P02789	gi|1351295	74	79551	6.85	2(0)	2(0)	2
	Serum albumin [Gallus gallus]	P19121	gi|45383974	69	71868	5.51	2(0)	2(0)	**
4	Serum albumin [Gallus gallus]	P19121	gi|45383974	997	71868	5.51	32(8)	23(7)	**
	PIT 54 protein [Gallus gallus]	Q98TD1	gi|46395491	82	52670	4.61	3(2)	2(1)	4
	Hemopexin (Gallus gallus)	P20057	gi|118106467	71	16191	5.61	2(1)	2(1)	**
5	serum albumin [Gallus gallus]	P19121	gi|45383974	360	71868	5.51	14(3)	10(2)	**
6	Fibrinogen beta chain	Q02020	gi|399491	708		7.18	33(13)	13(8)	30
	Chain C, Crystal Structure Of Native Chicken Fibrinogen	P14448	gi|8569623	146	47486	5.4	3(2)	3(2)	6
7	Chain C, Crystal Structure Of Native Chicken Fibrinogen	P14448	gi|8569623	370	47486	5.4	23(3)	10(2)	23
8	complement component 3d [Gallus gallus]	A6N9E0	gi|83655498	146	36820	9.11	4(1)	4(1)	11
	complement C3 precursor [Gallus gallus]	P04210	gi|45382303	103	185510	6.69	4(0)	4(0)	1
	Ig light chain precursor V-J region – chicken	*	gi|104728	50	22769	5.36	2(0)	2(0)	9
9	apolipoprotein A-I [Gallus gallus]	P08250	gi|211159	576	30673	5.58	22(7)	13(5)	44
	Ig light chain [Gallus gallus]	*	gi|212058	192	23868	5.34	5(2)	4(2)	17
	Ig light chain precursor V-J region – chicken	*	gi|104728	190	22769	5.36	5(2)	4(2)	17
	complement C3 precursor [Gallus gallus]	P04210	gi|45382303	149	185510	6.69	4(1)	4(1)	2
10	apolipoprotein A-I [Gallus gallus]	P08250	gi|211159	236	30673	5.58	19(0)	8(0)	29
	pre-fibrinogen alpha subunit [Gallus gallus]	P14448	gi|732995	215	56766	6.82	20(1)	6(1)	8
	Ig light chain precursor V-J region – chicken	*	gi|104728	60	22769	5.36	3(0)	2(0)	8

*name has since changed to Low-density lipoprotein receptor-related protein 1 (P98157)
**Mascot online database of results has moved this protein and original result.

Table 2.8: Proteins identified from 1DE (Figures 2.11 and 2.12) identified by Mascot *Gallus gallus* peptide database. Bands were excised from the gel trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates. Both NCBI and where Uniprot identifications are listed (immunoglobulins are not listed in Uniprot). The protein score is the sum of the highest ions score for each distinct sequence. The first number under matches is the count of MS/MS spectra that have been matched to peptides from this protein. The matches shown in the parenthesis indicating unique matches (scores above the significance threshold). The sequences column is the count of matches to distinct peptide sequences, the number in parenthesis is the count of sequences with significant matches (Matrixscience.com). The coverage percentage is proportion of the protein's entire sequence identified.

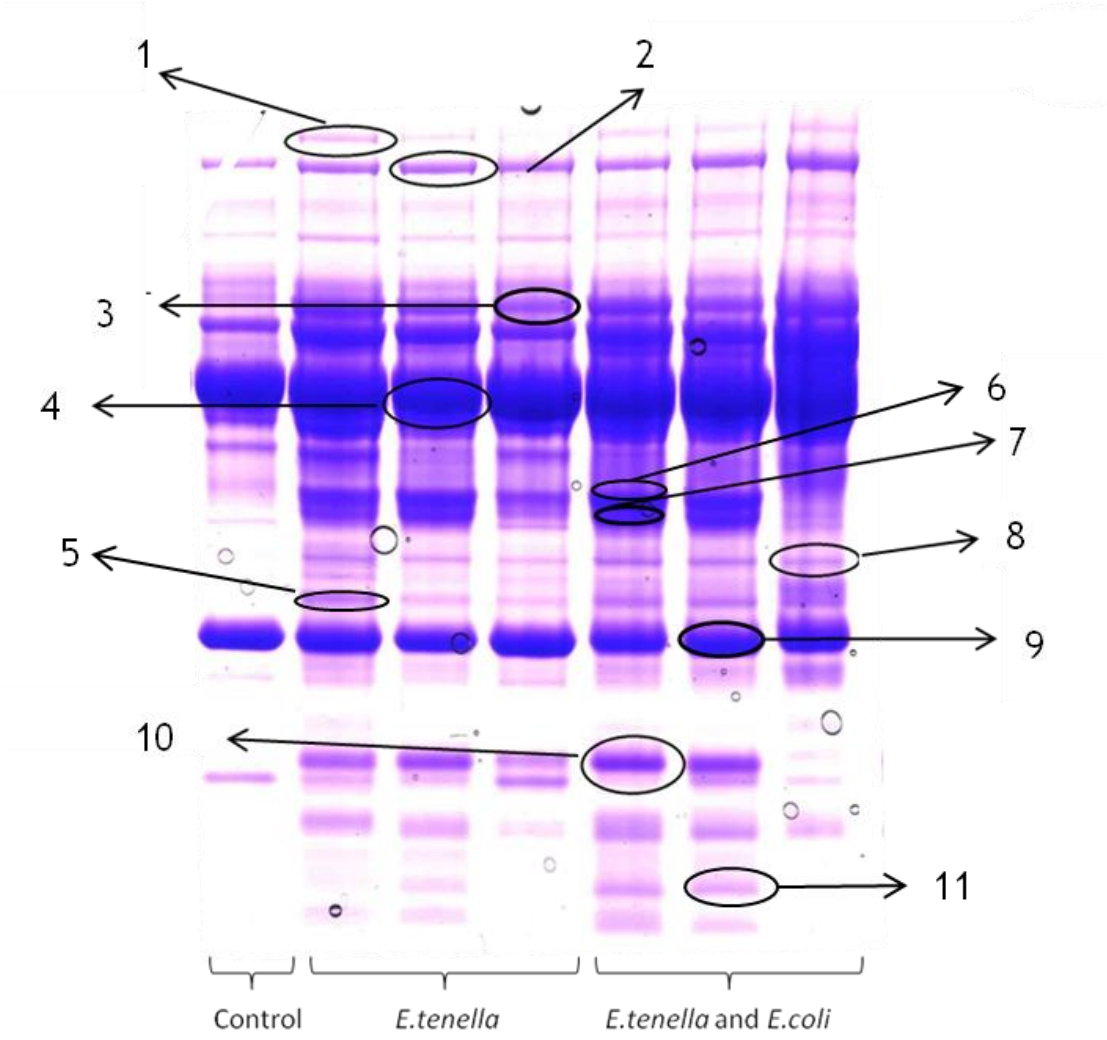


Figure 2.12: Excised bands identified with MS. The same, labelled, figure is shown in Figure 2.11. Results are detailed in Table 2.8. Separation was achieved using a 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124). Protein is stained with Coomassie blue G dye (Sigma Aldrich).

2.4 Discussion

2.4.1 Study 1: Acute phase proteins in culled and healthy broilers

Currently there is no published information available on the APP concentrations of broiler chickens from commercially sized poultry farms. There is a need for relevant reference ranges to compare experimental data and culled birds represent an accepted yet unnecessary economic loss to the poultry industry. This study determined the serum concentrations of PIT54 and Cp in healthy birds and birds culled for generalised ill health, namely stunted growth and lameness and found the concentrations to be significantly different, with culled birds having significantly higher concentrations of PIT54 and Cp.

The healthy birds had maximum serum concentrations of 0.055 g/L and 0.0980 g/L for PIT54 and Cp respectfully, with only two of the twenty healthy birds sampled having a serum Cp concentration within the detectable range of the assay, the rest fell below the minimal detectable limit (MDL) of the assay. The average PIT54 and Cp concentrations for healthy birds were 0.029 and 0.008 g/L respectfully. For PIT54 that is a 12 fold reduction on the average baseline of 0.36 g/L reported for healthy control birds in a number of studies (Georgieva *et al.*, 2010; Millet, *et al.*, 2007; Nazifi, *et al.*, 2010; Nazifi, *et al.*, 2011). For Cp the healthy birds had an average Cp concentration that was 9 fold lower than the reported average baseline for Cp in healthy control birds (Disilvestro & Harris, 1985; Georgieva et al., 2010; Richards & Augustine, 1988).

The lower PIT54 and Cp concentrations seen in the healthy birds from this study compared to those cited in the literature could be due to the variation that will inevitably exist between farms, studies, birds, assaying method and the wide time scale over which these studies were conducted. The serum Cp concentration of the healthy birds was less than the MDL in all but two birds. For both PIT54 and Cp many of the samples did not reach the MDL of the assay which could also be why there is variation in the serum concentrations in this study and those cited in the literature.

The age of the birds could have a strong effect on the serum concentrations of APPs. The ages of the birds in this study were of various ages ranging from 12 – 25 days and similarly the ages of the birds from the literature vary considerably making comparisons problematic. Further studies would be needed to ascertain whether age effects APP serum concentrations.

In this study birds were culled due to lameness or stunting, routine non-specific conditions that result in a birds removal from a flock. When the culled group was sub-divided into lameness and stunted, differences in PIT54 concentrations between these groups were detected. Dunn post hoc analysis revealed that PIT54 was significantly different between healthy and the stunted culled birds specifically, with no significant difference noted between PIT54 serum concentrations of the healthy and lame culled birds. Analysis of the culled bird Cp concentrations revealed no differences between the two culled groups.

Previous studies measuring the APPs of birds with specific causes of lameness found that metabolic diseases that cause lameness, namely tibial dyschondroplasia and femoral head separation disorders did not show elevations in serum Ovt, because as well as inflammation, infection or tissue injury was needed in addition to inflammation to evoke a rise in OVT (Rath, *et al.*, 2009). The results of this study suggest that the lame birds were not undergoing an APR. The presence of two outliers however, exhibiting much higher PIT54 concentrations in the lameness group suggest that more than one disease is responsible for the lameness, or that the birds were at differing stages of a disease process, with one disease or stage resulting in an acute inflammatory response, as evidenced by the two high PIT54 outliers, one of which is also produces the single high Cp outlier for lameness. The stunted birds were shown to be undergoing an inflammatory response consistent with the presence of an APR the causes of which were unknown but likely to be associated with the stunting.

2.4.2 Study 2: The effect of gait abnormality and NSAIDs on plasma acute phase protein concentration and plasma proteome.

In a second study conducted in collaboration with SRUC into gait scored broilers from commercial farms, it was found that the APPs PIT54, Cp and Ovt were all

significantly associated with gait, with GS3 birds having significantly higher Cp ($p \leq 0.001$), PIT54 ($p \leq 0.05$) and Ovt ($p \leq 0.05$) than GS1 birds. This study, unlike the previous smaller study, indicated that lameness was associated with an APR which significantly elevated all three APPs. The larger numbers of birds used in this study (GS3 n=68, GS1 n=64) compared with the smaller study (healthy n=20, culled n=18) gives increased statistical weight to these results. In addition the protocol based assessment of gait with an associated score is a more objective measure of lameness than that used in the first trial and may also have had an influence on these increased APP found with increasing GS. These results indicate that increased APP expression is associated with poor GS in broilers. Within the groups there was considerable variation in the APP concentrations which may indicate that sub-populations exist related to differing aetiologies of gait defect. All birds in this study were potential hosts to a wide variety of pathologies and conditions that cause leg weakness and affect gait.

There were no significant associations between the APP concentrations and broiler breed and although no significance was identified between weight and gait score, Cp was significantly associated with weight with heavier birds from both GS1 and GS3 tending to have higher Cp concentrations ($P \leq 0.05$). Higher Cp in the heavier birds may indicate that increasing weight results in a series of events, inflammatory in nature, that result in higher plasma Cp concentrations. Alternative explanations for this finding may lie in the causes of the increased bodyweight in this group of birds such increased feed intake.

Multiple studies have shown that both basal concentrations and acute phase concentrations of APPs can be affected by a variety of dietary supplements. Plasma Cp concentrations can be modulated by dietary copper concentrations (Song, *et al.*, 2009) and supplementations such as conjugated linolenic acid can affect the serum concentrations of APPs including Cp during experimentally induced APR (Takahashi, *et al.*, 2002). Protein supplementation can also illicit changes in the circulating concentrations of APPs. Takahashi, *et al.*, (1995) found birds fed a high protein diet had higher plasma alpha-1-acid glycoportein (AGP) concentrations than those fed a low protein diet. Those on the high protein diet went on to generate smaller increases in AGP as a result of LPS challenge than the low protein group. Notably feed restriction has been shown to have no effect on APP concentrations (Najafi, *et al.*, 2015). The significant

association between bodyweight and Cp concentration may be an inflammatory consequence of increase bodyweight, but this relationship requires further investigation. The findings of the aforementioned studies showing how the APR can be effectively modulated by changes in diet cannot be overlooked given the association between bodyweight and feed intake.

When comparing the proteomic profiles of the acute phase reactive plasma (APRP) and the non-APRP of GS3 and GS1 birds, differences were found in a number of regions with many spots differing in presence and intensity. Many of the proteins identified in the gels were found previously in the chicken serum proteomes detailed by Huang *et al.* (2006) and Gilbert *et al.*, (2011). The protein identifications reveal that a number of APPs were present in the proteomes of both acute phase and non-acute phase plasma (APRP/non-APRP) from GS1 and GS3 birds. Mass spectrometry and peptide fingerprinting identified the APPs OVT, PIT54, Hpx, AGP and Fb. Ceruloplasmin was not identified on any of the 2DE gels. The increased immunoglobulin (Ig) in the APRP from the GS3 birds together with the higher intensity of the Ovt spots and a well defined AGP spot that was either faint or completely absent in the non-APRP gels is supportive of the assay results that also indicate that an acute inflammatory reaction is taking place in the GS3 birds. Further proteomic work looking at the effects of lameness on the proteome would benefit from the removal of albumin and immunoglobulin as these are dominant proteins within the serum/plasma proteomes and their removal would allow other less abundant proteins to be identified more easily. A quantitative proteomic approach would also be of value in investigation of the APP in the chicken samples.

Birds in this study were designated into one of three groups: those injected with saline and two groups that receiving 2mg/kg and 5mg/kg of the NSAID meloxicam intramuscularly. Ovotransferrin was the only APP to be affected by meloxicam, and only at the higher dose. Both Cp and PIT54 concentrations remained unaffected by the administration of meloxicam. The literature suggests that at therapeutic doses, in animals not undergoing an inflammatory reaction, NSAIDs are unlikely to induce an increase in APP expression. There are numerous studies of inflammatory diseases that investigate the effects of NSAIDs on APPs concentrations and the results are variable. Lewis, *et al.*, (1998) for example found the NSAIDs to increase haptoglobin and Cp in experimental

models of rats with inflammation, whereas Bennett *et al*. (2013) found no changes in serum CRP in osteoarthritic dogs treated with NSAIDs. What has been established is that suprapharmacological doses of NSAISDs will affect the integrity of the intestine with the resultant translocation of bacteria and toxins into the systemic circulation which will stimulate an APR with resultant rises of APPs (Tugendreich, *et al*., 2006). Meloxicam is used, off license, in back yard chickens at a dose of 0.2-2mg/kg. At the 2mg/kg dose this study showed no significant increase of the measured APPs. At 5mg/kg, Ovt was shown to be significantly higher. It is highly probable that at this dose, the meloxicam had deleterious effects on the gastrointestinal tract – a recognised side effect of NSAIDs causing the increase in the moderate APP Ovt via the mechanism described above.

2.4.3 Study 3: Serum Ovotransferrin and Ceruloplasmin in chickens infected with *Escherichia coli* and *Eimeria tenellla*

The results of the study investigating the serum concentrations of Ovt and Cp during infection with *E. tenella* and *E. coli* revealed that *E. tenella* infection alone did not significantly increase the Ovt serum concentrations and that duel infection with *E. coli* was needed to evoke a significant rise in Ovt. This result contrasts with Rath *et al.,* (2009) who found Ovt to increase significantly with *E. tenella infection* alone. This Eimeria species colonises the caecal pouches causing haemorrhaging and localised pathological changes that are amongst the most serious of the Eimeria infections, yet despite this a concurrent infection of *E. coli* was also needed to evoke a significant rise in Ovt.

Eimeria tenella infection alone did significantly increase Cp concentrations as did the combination *E. tenella* and *E. coli* infection ($p \leq 0.01$ and $p \leq 0.05$ respectfully). These findings compare with Georgieva *et al.,* (2010) who also found Cp to significantly increase ($p \leq 0.05$) with both combination and *E. tenella* infection alone. Georgieva *et al.,* (2009) also found the APP Fb increased during *E. tenella* infection, yet like Ovt, PIT54 only significantly increased during a combined infection of *E. tenella* and *E. coli* and not *E. tenella* alone. This study also found that *E. coli* when administered alone did significantly increase PIT54

and Cp. The results of Georgieva *et al.,* (2010) and this study are suggestive of *E. coli* being a more effective stimulant of the APR.

When *E. tenella* infection causes an APR, the aetiology probably follows a similar course to the suprapharmacological doses of NSAIDs. *Eimeria tenella*, compromising the integrity of the intestinal barrier, results in the translocation of intestinal bacteria into systemic circulation with a resultant APR. Furthermore, a number of factors that have been shown to affect the outcome of naturally occurring Eimeria infections could also affect the results during experimental infections. Genetic factors and the interference of simultaneous infection with different Eimeria species and interactions with other pathogens such as viruses and enteric bacteria will determine the severity of the disease (Vermeulen, *et al*. 2001). Managerial factors, like poor hygiene, presence of other animals on the farm, the use of in-feed medication and the occurrence of other infectious diseases in the present or previous flock all affect the spread of the disease and the severity of the infection (Graat *et al*., 1998) and as such will affect the severity and duration of an APR.

A control sample and three samples of serum from birds infected with *E. tenella* and four samples from birds infected with both *E. tenella* and *E. coli* were subjected to 1D SDS gel electrophoresis. A number of bands appearing differentially present between infected and control samples were identified as APPs: Ovt, Fb, Fn and apo-AI. The proteins Complement 3 an activator of the innate immune system and alpha-2-macroglobin (low-density lipoprotein receptor-related protein 1) a major plasma protein were also found to be differentially expressed between samples though further work would be needed to assess the significance of these preliminary results.

Chapter 3

The development of an ovotransferrin radial immunodiffusion assay

3.1 Introduction

The competitive ELISA described in chapter 2 is an ovotransferrin (Ovt) assay detailed by Narayan Rath (Rath *et al*. 2009; 2010, *pers comm*.), modified and used to determine the concentration of Ovt. After applying this assay to a number of sample sets including the ones detailed in chapter 2, it was apparent that the repeatability, as represented by both intra- and inter-assay co-efficient of variants (CVs) were unsatisfactorily high. The intra-assay CV was 14% and the inter-assay CV was 32%. The reagent used in this assay, namely the rabbit α-chicken transferrin antibody (Accurate Chemical AI-AG 8240) was also prohibitively expensive analysis of a large numbers of samples. The method was lengthy and required large numbers of repeated washes.

To address the problem of an inadequate assay method for Ovt, an apt APP target in chickens, a new assay was developed. A radial immunodiffusion (RID) assay, using an antibody produced against egg derived Ovt as an antigen, was developed and applied to a number of samples sets.

3.1.1 Radial Immunodiffusion

The presence of a ring, formed in gels from the precipitation reaction between an antigen and anti-serum, was observed by early immunoscientists with Ouchterlony (1949) the first to detail the relationship between precipitate and anti-serum concentration and exploit single radial diffusion for quantitative purposes (Ouchterlony, 1949; Mancini, *et al.*, 1965). A single-diffusion precipitation reaction is performed by incorporating one of the two partners of the reaction, usually the antibody into agarose gel at a uniform concentration, whereas the other reactant, usually the antigen is introduced into a well from which it is allowed to diffuse into the gel where it will react with the internal reactant. Radial immunodiffusion occurs when a gel is flat and the diffusion occurs radially starting from a circular well (Mancini, *et al.*, 1965).

Mancini *et al.*, (1965) comprehensively detailed RID for the purposes of identification and quantification of a known antigen from whole serum, describing the linear relationship between the area of the precipitate and the concentration of the antigen. This method has been widely applied for research and diagnostic purposes, though its popularity has decreased since other assay

methods such as ELISAs, automated analysers and more recently quantitative proteomic methods have developed. Despite this, the RID method remains robust, straightforward, cost effective and reliable and as such, a good technique to measure and evaluate Ovt in chickens.

3.1.2 Egg Ovotransferrin

Ovotransferrin (Ovt) is synthesised both in the liver and the oviduct and is secreted into circulation or incorporated into the egg white respectfully. The antibody used in the assay described and modified from Rath, *et al*. (2009; *pers comm*. 2010) utilises rabbit α-chicken transferrin antibody (Accurate Chemical AI-AG 8240), a polyclonal antibody raised against chicken serum Ovt. Note that the difference between Ovt and transferrin is solely phraseologic, with most authors and databases such as Uniprot differentiating between types of transferrin and recommending the use of chicken ovotransferrin for the protein in an egg or in serum. Egg Ovt is available in a purified form (Conalbumin Sigma C0755) as a crystalline power derived from egg white. This was used as an antigen for antibody production.

3.1.3 Aims of study

The aims of this study were to produce an antibody using egg Ovt as an antigen, validate a RID assay and apply this assay to a number of studies to establish whether Ovt is a suitable APP target in chickens by applying it to a series of studies. Ovotransferrin was selected as an APP on which to focus further as it is a moderate APP in chickens. The availability of purified egg Ovt for use as an antigen for standards and antibody generation readily enables further assay development. It was hypothesised that the use of egg derived Ovt would enable the production of antibody that also cross reacts to chicken serum Ovt and that this antibody could be used to develop an assay that could be applied to a number of studies. The results of these studies are detailed in Chapters 4 and 5 where Ovt is measured alongside other APPs.

3.2 Materials and Methods

3.2.1 Reagents

Chemicals and materials used were obtained from Sigma Chem. Co. Poole, UK and Bio-Rad Laboratories LtdHemel Hempstead UK. Unless otherwise stated. Double distilled water was used throughout.

3.2.2 Egg Ovotransferrin

Purified egg Ovt (Conalbumin Sigma C0755) was diluted with dH_2O to a concentration of 5mg/ml and 1.25mg/ml and, alongside acute phase (AP) and non-acute phase (NAP) serum diluted 1:10, separated on a 1DE gel using the method describe in Chapter 2, Section 2.2.2.1. Pig serum and bovine foetal albumin (BFA) diluted 1:10 were also included on the gel. The gel was run in duplicate, enabling a western blot to be performed as described in Chapter 2, Section 2.2.2.2. The western blot used rabbit α-chicken transferrin antibody as the primary antibody diluted 1:5000 and goat α-rabbit IgG HRP (Ab 6721) as a secondary antibody diluted 1:20,000. Bands from the egg Ovt lanes were removed from the 1DE gel and trypsin digested, the proteins identified using MS as described in Chapter 2, Section 2.2.2.3.

3.2.3 Sheep α-chicken ovotransferrin antibody

To raise the antibody against egg Ovt, 1mg of egg Ovt dissolved in sterile saline together with 1.25ml of Freund's Complete Adjuvant in an emulsion of 2% Tween 80 and saline was injected into a sheep and bleeds recovered on a monthly basis thereafter. Subsequent boosters employing Freund's Incomplete, were delivered monthly 7 days prior to the bleed (Scottish National Blood Transfusion Centre, 21 Ellen's Glen Road, Liberton, Edinburgh).

3.2.4 Immunoelectrophoresis (IEP)

A 1% agarose gel was prepared by dissolving 0.17g of agarose (sigma A9539-log RK0015) in IEP buffer (0.08M Tris . 0.485mM Calcium lactate . 3.076mM Sodium azide . 0.02M Tricine). The liquid agarose was poured on an agarose film (Sigma Aldrich electrophoresis film for agarose gels b#110EO264-100EA), left to set at

room temperature before chilling at 4ºC for 15 minutes. 'Wells and troughs' were punched into the gel for samples (wells) and antibody (troughs) to go into.

To the sample wells 5µl of chicken serum either neat or diluted 1 in 4 was added. On each gel 5mg/ml egg Ovt was added to a well. To all wells 1µl of 1% bromophenol blue (BDH chemicals Ltd Prod 4430535534605) was added. The gel was run at 75V for 1.5 hours or until the bromophenol blue had migrated towards the anode but no further than the troughs end. The gel was transferred gel side up to humidity chamber. To the troughs, 150µl of antibody was added and the gels left at room temperature for 48 hours.

Following the incubation the following wash protocol was undertaken. The gel was washed in 0.09% NaCl overnight, and the following day pressure dried, to remove all the liquid from the agarose. The gel was washed in 0.09% NaCl for an hour and pressure dried to remove liquid from the gel. This was repeated once further. For the fourth and final wash the gel was washed in dH_2O for 1 hour, pressure dried and left to air dry over night. The following day the gel membrane was stained with Coomassie blue G dye (B0770, Sigma-Aldrich, Dorset, UK), prepared as 0.1% (w/v) Brillant Blue G, 25% (v/v) methanol, and 5% (v/v) acetic acid, for 20 minutes and de-stained in 10% (w/v) acetic acid in 25% (v/v) methanol for 1 hour. The gel was left to dry overnight. To determine the antigenicity of the sheep α-chicken ovotransferrin antibody and compare to the rabbit α-chicken transferrin antibody a series of IEP were performed.

3.2.5 Western blot analysis

To compare the reactivity of the antibodies to Ovt, western blots following 1DE were undertaken as previously described (Chapter 2, Sections 2.2.2.1 - 2.2.2.2). Three acute phase samples (AP) and three non-acute phase samples (NAP) were run alongside egg Ovt (1.25mg). The primary antibodies rabbit α-chicken transferrin (Accurate Chemical AI-AG 8240) and sheep α-chicken Ovt were used at a concentration of 1:200. Secondary antibodies, goat α-rabbit IgG HRP (Ab 6721) and rabbit α-sheep IgG HRP (Ab 6747) were diluted 1:20,000.

3.2.6 Mass spectrometry

From the 1DE gel selected bands of interest were removed, trypsin digested and the proteins identified using MS (Chapter 2, Section 2.2.2.3). Precipitates from the IEP membranes also underwent identification by the same method, by cutting the membrane and trpysin digesting the proteins contained within the precipitation arc.

3.2.7 Radial immunodiffsion (RID)

A single radial immunodiffusion gel contains antibody within the gel resulting in the formation of a precipitation ring when the antigen is added to a punched well. A 1% agarose gel was made by dissolving 0.17g of agarose (sigma A9539-log RK0015) in 17ml TBS. To establish a suitable antibody concentration 5ml of dissolved agarose was added to a conical tube and brought to temperature in a water bath at 56˚C. Rabbit anti-chicken transferrin antibody was pipetted into each of the 4 tubes at concentrations of 284µg/µl, 142µg/µl, 71µg/µl and 35.5µg/µl. After gentle mixing at 56ºC each of the agarose mixtures was poured onto a gel membrane and allowed to set at room temperature before transferring to a cold room where they remained for 15 minutes. Six wells were punched into the gels into which 8µl of Ovt standard at 5mg, 1.250mg/ml and 0.3125mg/ml, two AP and one NAP serum samples were added. The RID gel was incubated in a humidity chamber overnight at room temperature. The gel underwent the same washing and staining process described in Section 3.2.4. The resulted ring diameters were measured and a standard graph produced from which the Ovt concentration of the samples could be determined.

3.2.8 Ovotransferrin radial immunodiffusion assay

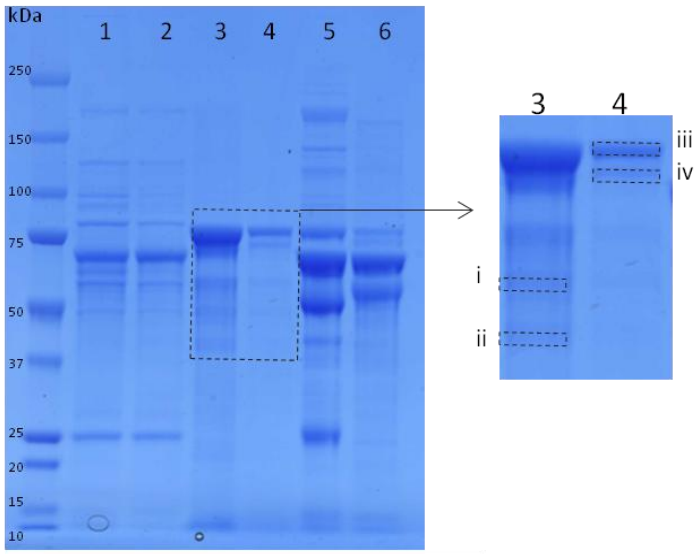
To establish whether there was a difference in precipitation ring between the rabbit α-chicken transferrin antibody and the sheep α-chicken Ovt antibody a number of RIDs were undertaken to establish whether this method could be used as an assay and to validate the sheep α-chicken transferrin antibody Further RIDs comparing the two antibodies determined that the sheep α-chicken Ovt antibody produced good precipitation rings. To determine the optimal antibody concentration of the sheep α-chicken Ovt antibody, four mini RID gels were

produced, with 100μl, 150μl, 200μl and 250μl of sheep α-chicken Ovt antibody added to 20ml of agarose, with 200μl found to be the optimal for clear ring precipitate formation. For assay validation, the RID assay was applied to a sample group (n=44) and intra and inter assay CVs determined.

3.3 Results

3.3.1 Egg Ovotransferrin

To investigate the protein content of the egg derived Ovt (Conalbumin Sigma C0755) prior to its use as an antigen for antibody production a 1D SDS gel and western blot using rabbit-α-chicken transferrin was undertaken (Figure 3.1).



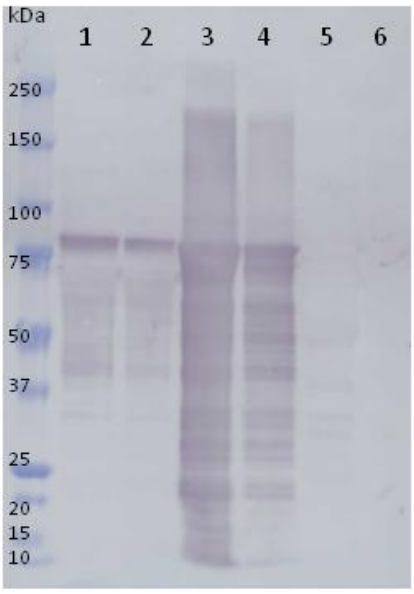


Figure 3.1 a. **Figure 3.1 b.**

Figure 3.1: 1D SDS gel: Proteins are separated with 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124) and stained with Coomassie blue G dye (Sigma Aldrich). Lane 1 and 2 contain chicken acute phase and non-acute phase serum respectfully (Figure 1a). Lanes 3 and 4 contain egg Ovt at concentrations of 5mg/ml and 1.25mg/ml respectfully. Lane 5 contains acute phase pig serum and lane 6 bovine foetal albumin. The bands extracted for mass spectrometry identification are identified in the highlighted area to the right: bands i-iv. A western blot using rabbit α-chicken transferrin was undertaken on a duplicate gel and is detailed in figure 1 b.

Figure 3.1 compares chicken acute phase serum with the purified egg Ovt (Figure 3.1.a) with the western blot (Figure 3.1.b) showing high affinity of the rabbit α-transferrin antibody with the egg Ovt as well as the serum Ovt. To identify proteins within the main double band at 75 and 76 kDa and also the lower molecular weight proteins, bands i – iv were excised from the 1D SDS PAGE gel

and identified using MS. The MS results identified Ovt as the protein present in all bands with the additional identification of ovalbumin in band i. (Table 3.1).

	Name	Locus	Uniprot accession	Score	Mass Da	PI	Matched	Sequence	Protein coverage
i	Ovt	TRFE_CHICK	PO2789	401	79551	6.85	41(12)	21(11)	43%
	Ovalbumin	OVALX_CHICK	PO1013	87	263331	5.1	5(1)	4(1)	18%
ii	Ovt	TRFE_CHICK	PO2789	593	79551	6.85	56(21)	36(17)	47%
iii	Ovt	TRFE_CHICK	PO2789	604	79551	6.85	50(18)	28(14)	43%
iv	Ovt	TRFE_CHICK	PO2789	481	79551	6.85	41(10)	28(8)	41%

Table 3.1: Mascot results of bands i-iv removed from lanes 3 and 4 of SDS PAGE gel in Figure 3.1. a. Bands were excised from the gel trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates. Both NCBI and where Uniprot identifications are listed (immunoglobulins are not listed in Uniprot). The protein score is the sum of the highest ions score for each distinct sequence. The first number under matches is the count of MS/MS spectra that have been matched to peptides from this protein. The matches shown in the parenthesis indicating unique matches (scores above the significance threshold). The sequences column is the count of matches to distinct peptide sequences, the number in parenthesis is the count of sequences with significant matches (Matrixscience.com). The coverage percentage is proportion of the protein's entire sequence identified.

3.3.1.1 Immunoelectrophoresis

Immunoelectrophoresis was used to compare both antibodies and their reactivity with chicken serum diluted 1:4. Both antibodies precipitate protein in the serum with the exact mobility as the egg Ovt. In addition the sheep α-chicken Ovt antibody precipitates a second arc that is more mobile than the first and clearly visible in all the samples except the egg Ovt (Figure 3.2). Further work revealed that when serum is added without dilution the rabbit α-chicken transferrin antibody also precipitates a second arc that, although not identical to the second arc seen with the sheep α-chicken Ovt antibody, also shows continuity with the main arc (Figure 3.3). This indicates that the protein within the second arcs share immunological identity to the main arcs, and has increased sensitivity to the sheep α-chicken transferrin antibody. The second arcs differ between the

antibodies, with the second arc of the sheep α-chicken Ovt antibody having increased mobility relative to the second arc precipitate of the rabbit α-chicken transferrin antibody and as such could be the result of different degradation products of Ovt.

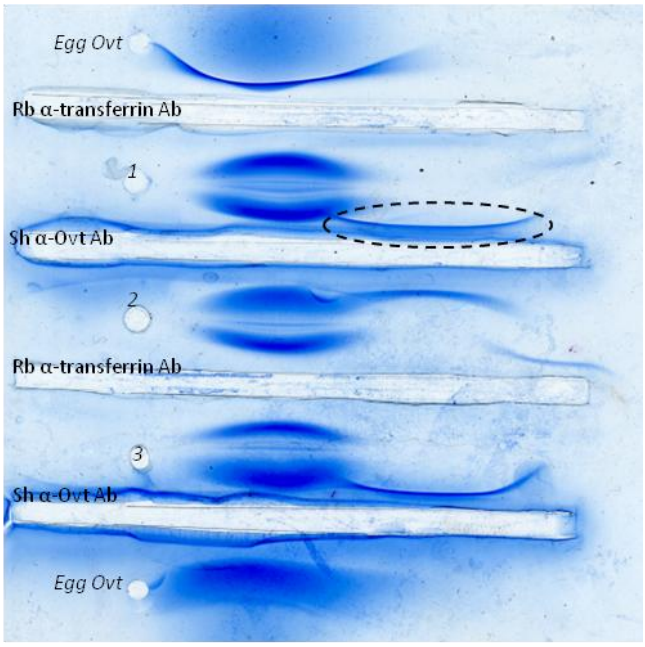


Figure 3.2: IEP gel (1% agarose) comparing the two antibodies. Rabbit α-chicken transferrin and sheep α-chicken Ovt antibodies and their reactivity to chicken serum and egg Ovt antigen are compared. The area highlighted is the second arc, visible only with the Sheep α-chicken Ovt antibody.

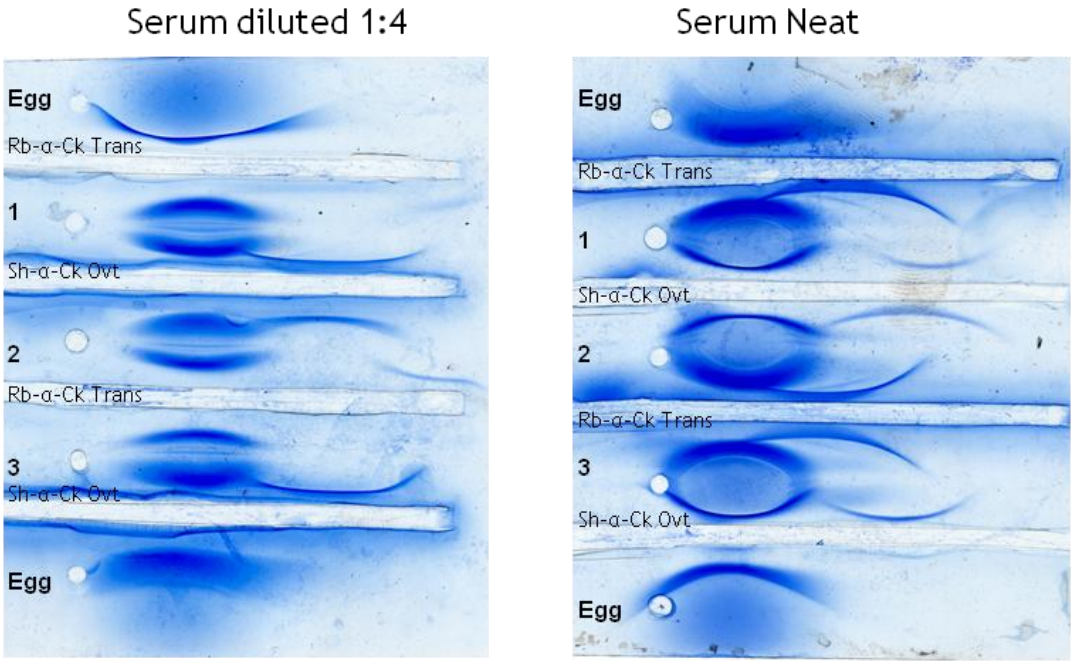


Figure 3.3: IEP gels (1% agarose) to compare rabbit α-chicken transferring and sheep α-chicken Ovt antibodies on undiluted and diluted serum.

3.3.1.2 Mass spectrometry

To investigate the nature of the second arc produced with the sheep α-chicken Ovt antibody and determine whether it was an isoform, fragment or degradation product of the main Ovt arc, MS was used to identify the proteins within the precipitate arcs. From the IEP gel eight sections were excised (on plastic membrane) for trypsin digestion (Figure 3.4). Table 3.2 details the proteins identified within each precipitate. The proteins identified in the precipitates as well as including Ovt, which is the most abundant, also include other major plasma proteins including apo-AI, Fb and ovoinhibitor. The most probable cause of the other proteins identified by mass spectrometry within the precipitates is that the washing of serum from the IEP gel was incomplete which together with a highly sensitive proteomic method of protein identification results in the identification of other plasma protein that remain on the IEP gel.

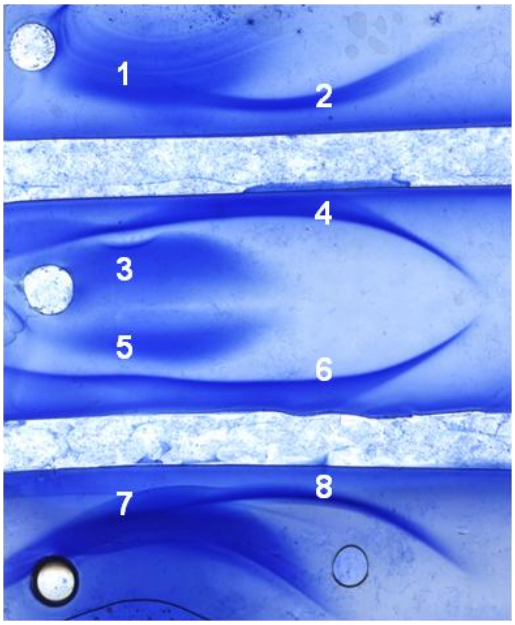


Figure 3.4: IEP (1% agarose) membrane with excised areas labelled. Gel contains sheep α-chicken Ovt antibody (trough) and serum samples (wells). Proteins identified are detailed in Table 3.2.

Arc	Protein ID	NCBI Accession	MW (Da)	Pi	Peptides matched	Sequences	Coverage	Score
1	Ovotransferrin	gi|1351295	79551	6.85	63(26)	29(16)	42	705
	Chain A, Crystal Structure Of Aluminum-Bound Ovotransferrin	gi|83754919	77518	6.70	63(26)	29(16)	44	705
	ovotransferrin CC type	gi|71274077	79575	7.08	57(22)	28(15)	41	660
	Fibrinogen beta chain	gi|399491	53272	7.18	22(9)	16(8)	37	291
	Chain C, Crystal Structure Of native Chicken Fibrinogen	gi|8569623	47486	5.40	17(7)	12(4)	26	175
	pre-fibrinogen alpha subunit	gi|732995	56766	6.82	15(3)	8(3)	18	162
2	Apolipoprotein B precursor	gi|113206052	524519	8.51	14(1)	12(1)	2	138
	Titin isoform X3	gi|513193910	377391	6.02	49(0)	38(0)	1	97
	Ovoinhibitor	gi|513206786	59590	6.44	5(1)	4(1)	8	72
	Lysosomal-trafficking regulator	gi|513175724	433306	5.92	7(0)	7(0)	1	59
3	fibrinogen alpha chain isoform 1 precursor [Gallus gallus]	gi|429484490	83139	6.86	24(14)	12(7)	37	494
	Chain A, Crystal Structure Aluminum-Bound Ovt	gi|83754919	77518	6.7	53(18)	27(13)	38	409
	Ovotransferrin CC type	gi|71274077	79575	7.08	50(16)	26(12)	36	376
	Fibrinogen beta chain	gi|399491	53272	7.18	22(7)	15(7)	39	271
	Chain C, Crystal Structure Of Native Chicken Fibrinogen	gi|8569623	47486	5.40	18(8)	13(8)	31	185
4	Ovotransferrin (conalbumin)	gi|1351295	79551	6.85	22(4)	17(4)	27	98
	Chain A, Crystal Structure Aluminum-Bound Ovt	gi|83754919	77518	6.70	22(3)	17(3)	28	98
	Ovotransferrin CC type	gi|71274077	79575	7.08	22(3)	17(3)	27	83
	Ovoinhibitor [Gallus gallus]	gi|513206786	59590	6.44	6(3)	5(2)	63	73
	Chain C, Crystal Structure Of Native Chicken Fibrinogen	gi|8569623	47486	5.40	3(1)	3(1)	9	43
5	Ovotransferrin	gi|1351295	79551	6.85	62(33)	28(21)	41	717
	Chain A, Crystal Structure Aluminum-Bound Ovt	gi|83754919	77518	6.7	62(33)	28(21)	43	717
	Ovotransferrin BC type	gi|71274079	79588		28(29)	28(19)	44	651
	Fibrinogen alpha chain	gi|1706798	56766	6.82	24(9)	13(6)	26	322
	Fibrinogen beta chain	gi|399491	53272	7.18	20(12)	16(11)	44	303
	Apolipoprotein A-I precursor	gi|211146	30672	5.97	4(2)	4(2)	15	52
6	Ovoinhibitor [Gallus gallus]	gi|513206786	59590	6.44	2(1)	2(1)	*	83
	Apolipoprotein A-I precursor	gi|211146	30672	5.97	2(1)	2(1)	7	47
7	Chain A, Crystal Structure Aluminum-Bound Ovt	gi|83754919	77518	6.7	71(29)	25(16)	41	963
	ovotransferrin CC type	gi|71274077	79575	7.08	68(29)	24(16)	38	954
	Ovotransferrin	gi|1351295	79551	6.85	70(28)	24(15)	36	898
8	Ovotransferrin	gi|1351295	79551	6.85	10(3)	9(3)	14	100
	Ovoinhibitor precursor	gi|71895337	59590	6.44	8(1)	6(1)	*	45
	Apolipoprotein A-I precursor	gi|211146	30672	5.97	3(1)	3(1)	10	39
	Apolipoprotein AI	gi|227016	28790	5.45	3(1)	3(1)	11	39

Table 3.2: Mass spectrometry results from IEP (Figure 3.4). *Coverage data not available. The IEP membrane containing specified arcs were cut out and the membrane containing precipitated protein trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus*. Both NCBI and where Uniprot identifications are listed (immunoglobulins are not listed in Uniprot). The protein score is the sum of the highest ions score for each distinct sequence. The first number under matches is the count of MS/MS spectra that have been matched to peptides from this protein. The matches shown in the parenthesis indicating unique matches (scores above the significance threshold). The sequences column is the count of matches to distinct peptide sequences, the number in parenthesis is the count of

sequences with significant matches (Matrixscience.com). The coverage percentage is proportion of the protein's entire sequence identified.

3.3.1.3 Fibrinogen and ovoinhibitor

With a view to eliminating Fb and ovoinhibitor as proteins precipitating with the sheep α-Ovt antibody to form the second more mobile arc two further steps were taken. For fibrinogen an IEP was undertaken using both plasma and serum. Figure 3.5 shows the IEP gel and no difference in the arc patterns is notable between the two sample types.

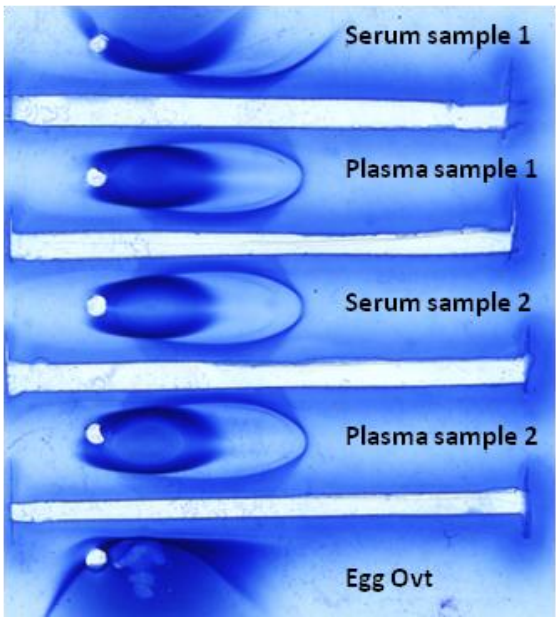


Figure 3.5: IEP membrane (1% agarose) with serum and plasma samples (wells) from the same individual chickens which is separated and precipitated with sheep α-chicken transferrin antibody (troughs).

Similarly an IEP using ovoinhibtor (as trypsin inhibitor from chicken egg white/type II-O partially purified ovomucoid containing ovoinhibitor, Sigma T9253) resulted in no precipitation arc and a western blot using the ovoinhibitor also yielded a negative result (results not shown). Ovoinhibitor was included as a sample on a RID gel with both antisera in the agarose gel and in one of the two wells into which it was added yielded a very faint ring was seen with sheep a-chicken Ovt (Figure 3.6).

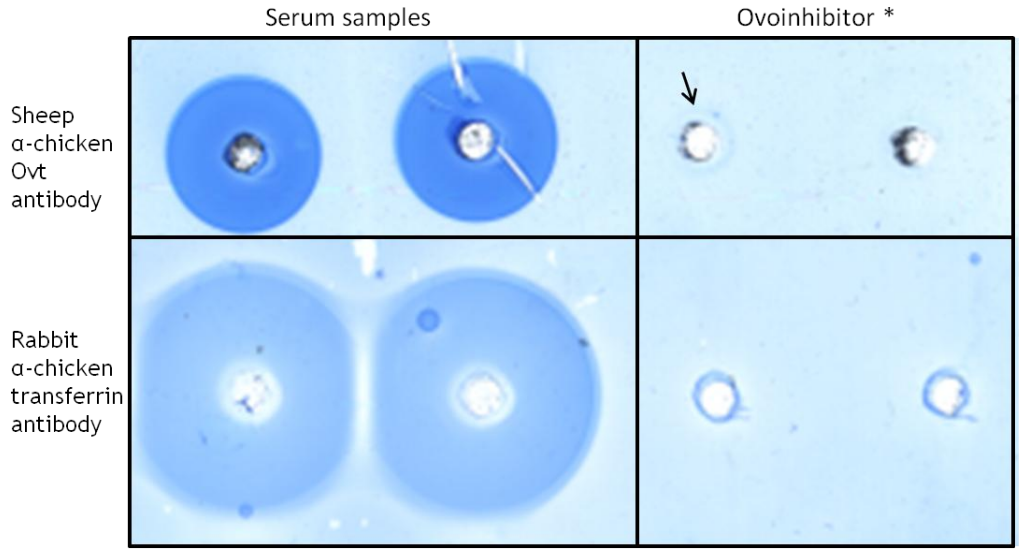


Figure 3.6: RID gel comparing serum and ovoinhibitor (*as trypsin inhibitor from chicken egg white/type II-O partially purified ovomucoid containing ovoinhibitor, Sigma T9253) precipitation rings with sheep α-chicken Ovt and rabbit α-chicken transferrin antibodies.

3.3.1.4 Validation of specificity of Sheep α-chicken ovotransferrin antibody

To determine the specificity of the antiserum for Ovt RIDs using the sheep α-chicken Ovt antibody with serum samples showed clear merging of the outer precipitation ring of three samples of egg Ovt (standards) and the serum Ovt within three samples (Figure 3.7). The merger of the ring round a serum sample and purified egg Ovt demonstrates immunological identity and confirms the RID is specific.

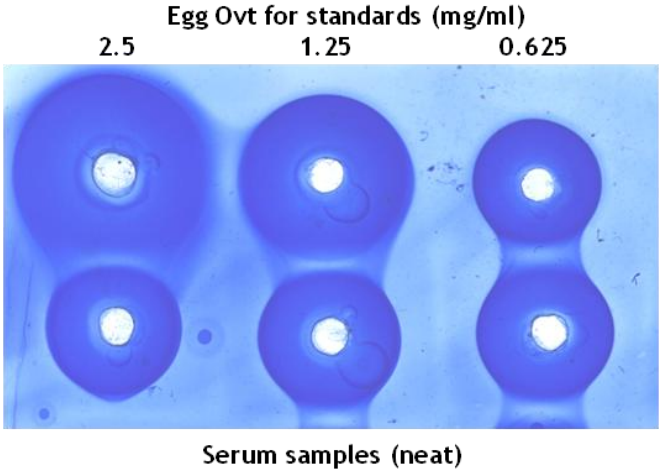


Figure 3.7: Ovt rings from three egg standards (top row) and three serum samples (bottom row) showing merging of precipitation rings, in an RID using the sheep α-chicken Ovt.

3.3.2 Ovotransferrin radial immunodiffusion assay

Initial studies applying the rabbit α-transferrin antibody to a RID assay method established the optimal concentration at which the antibody should be diluted in agarose (Figure 3.8). A volume of 100µl in 5ml of agarose (a concentration of 142µg/ml) produced the most optimal rings for measurement with this antibody.

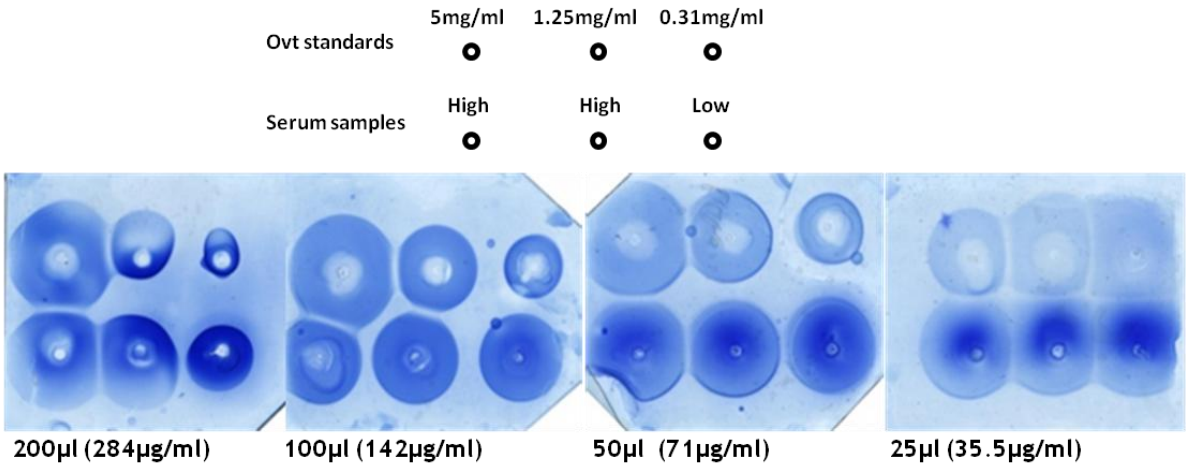


Figure 3.8: Mancini RID gel of three samples and the Ovt standards and different rabbit α chicken transferring antibody concentrations. The gel layout is detailed above.

To optimise the dilutions of the serum a further RID was undertaken using 1:4 and 1:8 serum dilutions. OVT standards were added starting at 1.250 mg/ml and serially diluted four fold. Figure 3.9 details the diluted serum and standard rings. Measuring the ring diameters and plotting diameter against concentration to produce a standard curve (Figure 3.10).

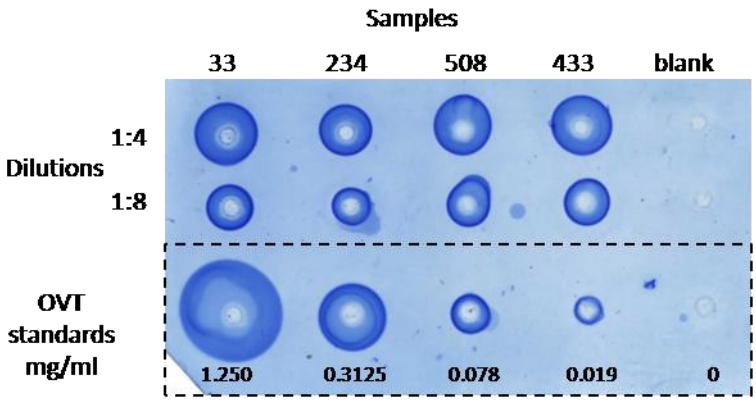


Figure 3.9: RID with four diluted serum samples (top row of rings) and OVT standards (bottom row of rings). The serum is serially diluted.

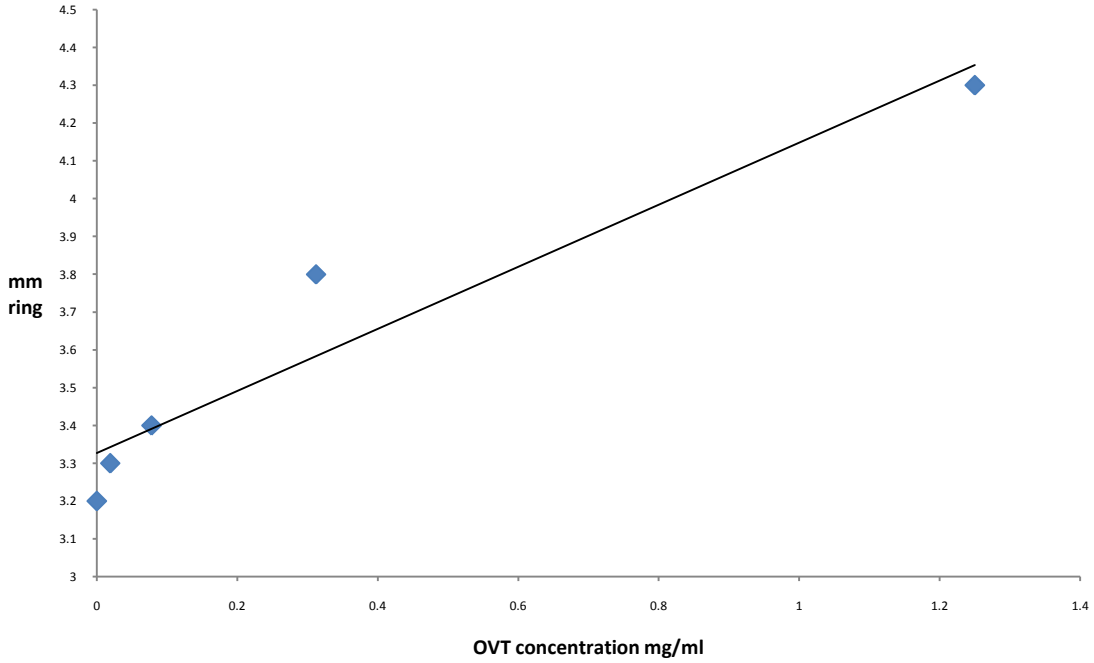


Figure 3.10: Standard curve of OVT produced from RID ring diameters in previous figure.

To compare the commercial rabbit α-chicken transferrin and the sheep α-chicken Ovt antibodies, two RIDs using the same standard concentrations as previously used, but with undiluted serum samples were produced and are shown in Figure 3.10. The concentration of antibody for the sheep α-chicken Ovt RID is less than optimal and further work to optimise the concentration of this antibody for RID was undertaken. The two low samples (NAP) and the two high samples (AP) show good repeatability and a clear difference in ring size between the two acute phase status samples.

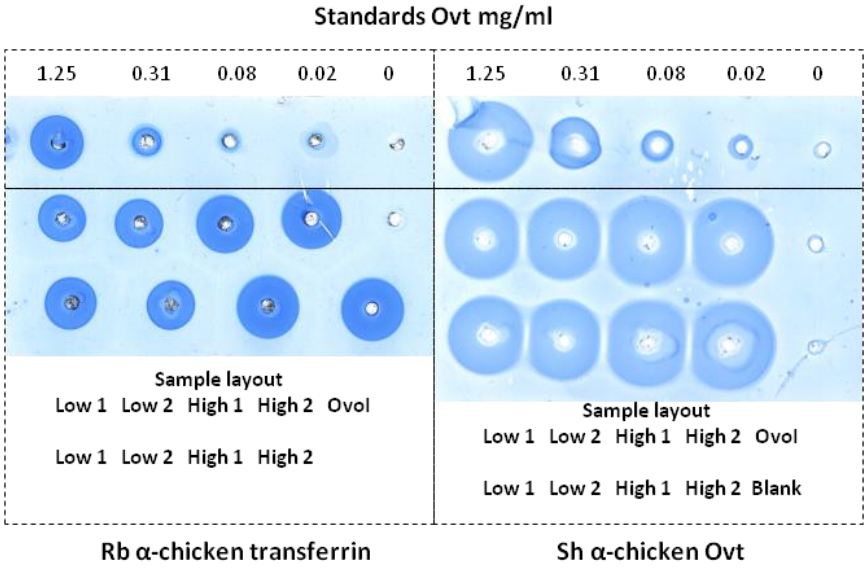


Figure 3.11: RIDs of Ovt standards and four samples using two antibodies. Both gels also contain ovoinhibitor (Ovol) as highlighted in Figure 3.6.

Using the standard ring diameters standard curves for both antibodies were produced (Figure 3.12) from which Ovt concentrations of four chicken samples: two acute phase (high 1 and 2) and two non-acute phase (low 1 and 2) were calculated. Table 3.3 contains calculated concentrations and CVs to compare the two antibodies.

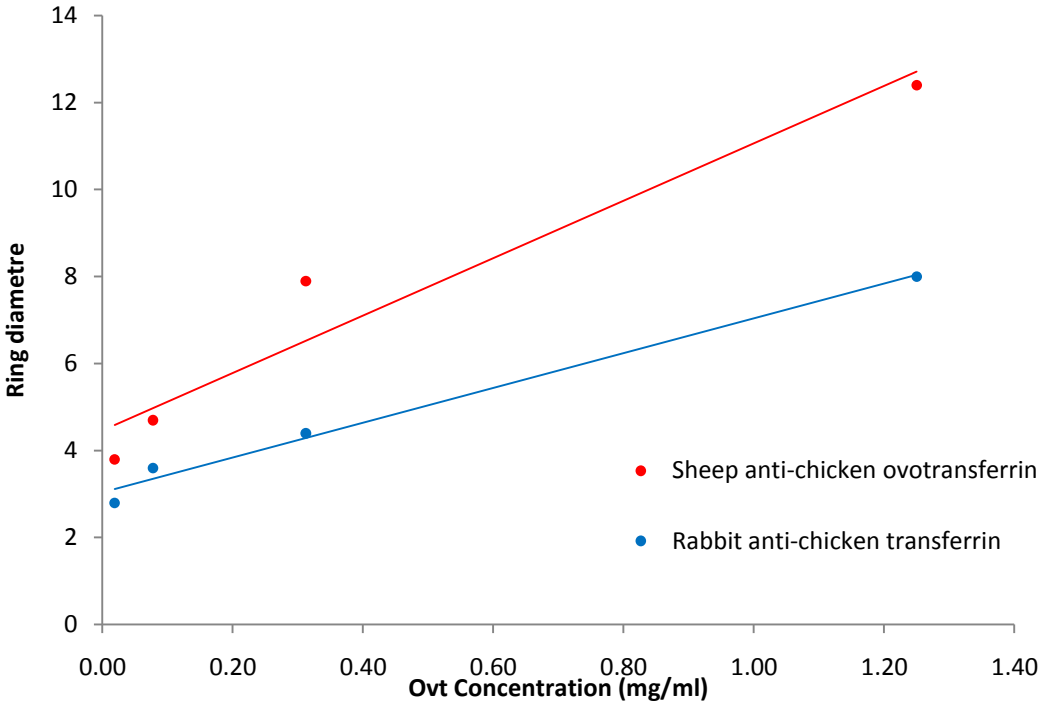


Figure 3.12: Standard curve of RID using sheep α-chicken Ovt and rabbit α-chicken transferring antibodies.

Antibody	Sample	Ring diametre	Calculated Ovt conc. mg/ml	Average	SD	CV
Rabbit anti-chicken transferrin	Low 1	7	0.99	1.05	0.09	8.73
	Low 1	7.5	1.12			
	Low 2	7.3	1.07	1.07	0.00	0.00
	Low 2	7.3	1.07			
	High 1	8.9	1.48	1.51	0.04	2.44
	High 1	9.1	1.54			
	High 2	9.2	1.56	1.60	0.06	3.45
	High 2	9.5	1.64			
			Mean Intra-assay CV			**3.65**
Sheep anti-chicken ovotransferrin	Low 1	12	1.17	1.19	0.03	2.48
	Low 1	12.2	1.21			
	Low 2	12.5	1.27	1.27	0.00	0.00
	Low 2	12.5	1.27			
	High 1	13.5	1.48	1.49	0.01	0.99
	High 1	13.6	1.50			
	High 2	13.8	1.54	1.53	0.01	0.96
	High 2	13.7	1.52			
			Mean Intra-assay CV			**1.11**

Table 3.3: Calculated serum Ovt concentrations and CVs of high and low samples using RID. Low and high samples were ran in duplicate, and the average CV of all samples taken as total CV.

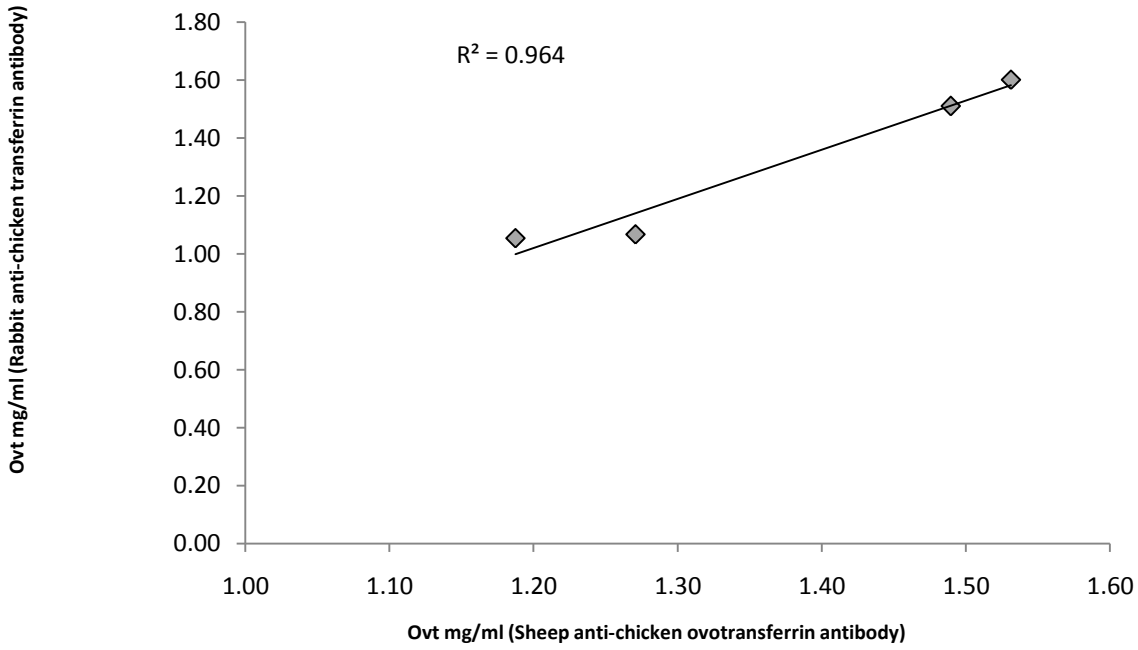


Figure 3.13: Correlation of calculated Ovt concentrations from four samples using two RID assay utilising two different antibodies.

For further validation that the sheep α-chicken ovotransferrin antibody is indeed measuring serum Ovt in the chicken, Ovt concentrations of samples using both antibodies were plotted against each other to produce an R2 value of 0.96 (Figure 3.13).

3.3.2.1 Assay Validation

Continuing with an RID method using only the sheep α-chicken Ovt antibody, a total of 44 samples were measured across 5 gels to validate the assay and determine its repeatability over a larger number of samples. Two quality control (QC) samples were used on each gel in triplicate. Standards were double diluted from 2.5 – 0.3mg/ml to produce a standard curve (Figure 3.14). The inter- and intra- assay coefficients of variants (CVs) were calculated. Table 3.4 contains the results of the intra-assay CV for each of the gels, calculated from the triplicate results of each sample.

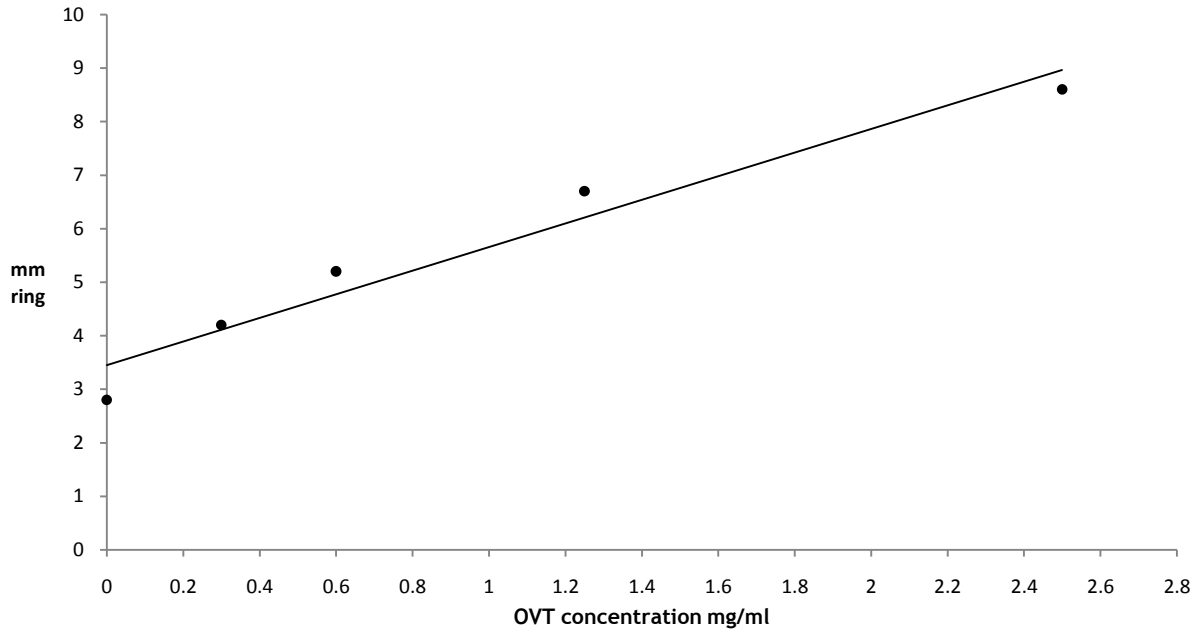


Figure 3.14: Standard curve using sheep α-chicken ovotransferrin, with standards 2.5, 1.25, 0.6 and 0.3 mg/ml. Standard curve appears more linear at these standard concentrations.

RID gel	Intra assay %CV
Ovt RID gel #11	**4.88**
Ovt RID gel #12	**8.30**
Ovt RID gel #13	**5.56**
Ovt RID gel #14	**9.62**
Ovt RID gel #15	**7.97**
Mean	**7.27**

Table 3.4: Intra-assay CV of replicate samples on each gel.CVs for each sample (ran in triplicate) were averaged to produce an intra-assay CV for that gel.

The inter-assay CV was calculated by calculating the standard deviation of each of the average Ovt concentrations for each of the QC samples from all five gels and calculating the CV. Table 3.5 details the results of the QC samples.

Gel	Sample	Average Ovt mg/ml	SD	CV
11	500	2.522	0.249	9.88
	493	2.325	0.038	1.63
12	500	2.016	0.057	2.83
	493	1.374	0.157	11.40
13	500	2.096	0.126	5.99
	493	1.641	0.068	4.12
14	500	2.115	0.068	3.21
	493	1.538	0.096	6.25
15	500	2.303	0.237	10.30
	493	1.842	0.237	12.88

Sample 500		**Sample 493**	
Gel	**Ovt mg/ml**	**Gel**	**Ovt mg/ml**
11	2.522	11	2.325
12	2.016	12	1.374
13	2.096	13	1.641
14	2.115	14	1.538
15	2.303	15	1.842
Mean	**2.210**	**Mean**	**1.744**
SD	**0.203**	**SD**	**0.366**
CV%	**9.197**	**CV%**	**20.999**

Table 3.5: Calculated inter assay CVs for QC samples. Quality control (QC) samples (high and low) ran on every gel, intriplicate used to produce a CV which was then averaged to produce a CV for the high and low QC, from which the mean CV was calculated.

3.4 Discussion

The competitive Ovt ELISA, detailed in Chapter 2, was found to be expensive, time consuming and have poor repeatability. Given the availability of the egg derived Ovt that could serve as an immunogen for antibody production, the initiative was taken to produce an antibody that would cross react with the serum Ovt for use in an assay with higher precision and allow multiple analyses to be undertaken as described in Chapters 4 and 5. Before using a commercial protein as an antigen it was important to assess its purity so that the antibody would be as specific as possible. Western blot analysis revealed that purified Ovt (Conalbumin Sigma C0755) consisted of a protein 75-76kDa that bound both antisera at the same molecular weight as serum Ovt and MS confirmed this to be Ovt. Bands present at lower molecular weights within the egg derived Ovt were also revealed to be Ovt. Ovalbumin, the major component of egg white (Awadé, *et al.*, 1994) was also identified in a minor band, with one significant match to a peptide sequence made, making it likely that this was a minor contaminant of the purified Ovt. These results confirmed that the preparation could be used as an immunogen for antibody production.

3.4.1 Validation of sheep α-chicken ovotransferrin antibody

Initial IEP comparing rabbit α-chicken transferrin and sheep α-chicken Ovt antibody revealed the presence of a second more mobile precipitation arc, the result of a more mobile protein forming a precipitate between the sheep α-chicken Ovt antibody and serum. The nature of the protein causing this second arc was investigated to determine whether it was an Ovt fragment or degradation product or a protein precipitating with a contaminant antibody within the sheep α-chicken Ovt antiserum. A contaminant was ruled out once the IEP was re-ran using undiluted serum, which revealed that the a similar arc was also present with the rabbit α-chicken transferrin antibody, meaning that the same or similar protein isoform or degradation product was also precipitating with the rabbit α-chicken transferrin antibody, albeit with less sensitivity, hence the need for more concentrated serum to identify it.

Mass spectrometry results of the IEP precipitation arcs yielded large volumes of data and identified Ovt in all but one arc. The sensitivity of the MS together with

the inability of the IEP gel membrane to be washed clear of all but the precipitated proteins meant that despite the sensitivity of this method, the specificity was limited as other proteins, not necessary from the precipitate were also identified. Given the large Mascot Score for Fb and the presence of the protein ovoinhibitor in two of the second arcs, these were singled out for further analysis.

The IEP to compare plasma and serum revealed no difference between the antibody reactions and excluded fibrinogen as a contaminant. Investigation of ovoinhibitor yielded mixed results. The IEP and western blot analysis reveal no arc or band respectfully, yet an RID well loaded with trypsin inhibitor from chicken egg white/type II-O partially purified ovomucoid containing ovoinhibitor (Sigma T9253) revealed a small faint ring. This finding is likely to be artefact (it was not identified in replicate wells) though further work, ideally using a more purified form of ovoinhibitor would be needed to confirm this conclusion.

It is highly likely that the second precipitation arc is an Ovt fragment which by the nature of its structure and glycosylation pattern is more mobile than the parent protein from which it is derived. Initial work on the purified egg Ovt identified a number of bands with lower molecular weights than the main Ovt bands, and these were identified as Ovt. The presences of the second arc, seen with both antibodies, together with the Ovt bands of differing molecular weight seen on 1D SDS PAGE and western blots reveal that other Ovt products and fragments exist that are as immunologically sensitive as the parent protein. These fragments, evident as the second arc, merge with the major precipitated protein in IEP which demonstrate that there is immunological identify with Ovt so that the antiserum can be used in immunoassay to measure this protein in serum. To further investigate the nature of the second precipitation arc and fully determine the differences between the Ovt seen in the main and second arcs, immunoaffinity separation and MS could be employed.

Further validation for the use of the sheep a-chicken Ovt came from the shared immunological identity with the egg Ovt which was observable when two RID rings, from egg and from serum merge together. This is indicative of a single protein precipitation product and was seen on numerous RID gels.

Further work to develop this assay for use on an automated analyser using an immunoturbidimetric format would allow increased throughput and widen the measurement of this APP into many other areas of poultry research. In its current form however it remains an economic and straightforward assay that is been utilised across many poultry research areas (Najafi *et al*., 2009; Shakeri *et al*., 2014; Najafi *et al*., 2015).

3.4.2 Validation of ovotransferrin radial immunodiffusion assay

Once the optimal dilutions of egg Ovt and antibody concentration within the agarose gel were determined, the results of samples measured using an RID assay using rabbit α-chicken transferrin antibody and an RID assay using sheep α-chicken Ovt antibody were correlated. The R2 value of 0.96 was calculated which adds further validity to the sheep α-chicken Ovt antibody and the RID assay. The inter- and intra- assay CVs for the samples measured were 15.0% and 7.3% respectively. Compared to the CVs for the competitive Ovt ELISA, which had inter- and intra- assay CVs of 32.0% and 14.9% respectively, the CVs for the Ovt RID have been halved and as such make this assay a more reliable, repeatable and precise measure of serum Ovt in chickens than the ELISA system as used in Chapter 2.

Chapter 4

The measurement of chicken acute phase proteins and identification and measurement of other biomarkers of the acute phase response using a quantitative proteomic method.

4.1 Introduction

Research on chickens APPs has largely focused on proteins previously identified in other veterinary species and humans, identifying and purifying an APP from plasma before measuring it in chickens during experimental infections. This approach, while successful in many respects has revealed that some APPs, for example (ovo-) transferrin do not respond in chickens as they do in mammals during an APR. Detailing the behaviour of known APPs in chickens during an APR is necessary as there are many APP, particularly the negative APPs that have received very little attention in chickens.

This can be hindered by the fact that for many chicken APPs, specific and validated commercial immunoassays are not available. The limited knowledge of chicken APPs and in some cases the low abundance in serum, even during an APR is problematic for assay development. Developing immunoassays is also costly, time consuming and slow. Moving away from immunoassays, proteomic approaches can be utilised not only to identify differentially expressed proteins between healthy and diseased animals but also quantify them. Applying quantitative proteomic techniques to the field of APP research in chickens would allow identification of the major APPs and confirm the presence and behaviour of other APPs in this species.

The major APPs in chickens are yet to be determined. The APPs serum amyloid A (SAA) and C-reactive protein (CRP) are major APPs in other species, and are used routinely in human and veterinary medicine. Serum amyloid A is a major candidate for a major chicken APP having been demonstrated to increase up to 1000 fold in earlier studies (Upragarin, 2005). Although a pentraxin gene has been identified in chickens there are no reports of CRP being purified and identified in chicken plasma or sera though there are studies that have measured CRP in chickens and cite commercially available kits. Conclusive identification and measurement of this protein in chickens is needed.

Other APPs targeted in this study are introduced here alongside SAA and CRP. In mammalian species the APPs apolipoprotein AI (Apo-AI) and transthyretin (Ttn) behave as negative APPs, their behaviour in chickens during an APR is unknown and as such these are appropriate targets in this study. Haemopexin (Hpx) is an established yet not widely measured APP in chickens while PIT54, Ovt and AGP

have been previously measured and are included here to complete the panel of APPs measured. Where these APPs have not been studied widely or at all in chickens, information has been gathered from mammalian studies with a view of contextualising these APPs in this study.

The quantitative proteomic method employed in this study identifies *all* differentially abundant proteins between acute phase and non-acute phase samples in chickens and will thus allow identification of other proteins that may be biomarkers of the APR in this species. Moving away from the "one rule fits all" approach to investigating the chicken APR and identifying and measuring other proteins that show differential abundance in serum during the APR will determine whether other, novel, biomarkers of the APR exist in chickens.

4.1.1 Serum Amyloid A

Serum amyloid A (SAA) is a major vertebrate APP and in most species the most sensitive protein of the APR. The SAA family of APPs comprises a number of differentially expressed apolipoproteins, associated with high density lipoprotein (HDL), including two isoforms of hepatic origin: SAA-1 and SAA-2, these are acute phase SAA proteins that can increase 1000 fold during an APR implying an important beneficial role in host defence (Eriksen *et al.*, 1993; Uhlar & Whitehead, 1999; Röcken & Shakespeare, 2002). In mammals a constitutive SAA (SAA-4) is present as a minor HDL apolipoprotein that comprises over 90% of SAA during homeostasis and is not significantly increased in acute phase serum (Landman, 1998; Röcken & Shakespeare, 2002). In mammals multiple SAA genes and proteins, strongly induced during an APR, have been described (Uhlar and Whitehead, 1999; Upragarin *et al.*, 2005). In chickens only one SAA gene has been identified (Ovelgönne *et al.*, 2001). Extrahepatic synthesis of SAA (isoform SAA-3) has been documented to occur in a number of mammalian tissues, including intestine, kidney, endocrine organs, synovial fibroblasts and macrophages (Uhlar & Whitehead, 1999). In avian species, SAA is primarily synthesised in the hepatocytes. Extraheptic synthesis of SAA has been shown to occur in duck lung tissue (Guo *et al.*, 1996) and chicken fibroblast-like synoviocytes (Upragarin *et al.* 2005).

Serum amyloid A appears to be highly conserved within mammalian species and is present in other vertebrates including birds and fish as well as invertebrates

(Santiago-Cardona *et al.*, 2003). This conservation of SAA together with the wide range of tissues where SAA is expressed and the dramatic induction of SAA production shortly after tissue damage, makes it evident that SAA plays an essential protecting role in the organism (Soler *et al.*, 2013).

4.1.1.1 Serum amyloid A structure and function

Analysis of human and murine circulating SAA primary sequences reveal it to be a hydrophobic, amphipathic protein consisting of a N-terminal helical bundle that comprises the 80% of the protein and a remaining potentially disordered C-terminus that might serve as ligand binding region (Stevens, 2004; Soler *et al.*, 2013). Modulating lipoprotein transport and metabolism during an APR appears to be a major function of SAA. Serum amyloid A allows cholesterol to remain in damaged tissues where it is needed for repair and regeneration of membranes and to transport cholesterol and clear lipid debris from bacteria and damaged areas of tissue (Eriksen *et al.*, 1993; Landman, 1998). During an APR, SAA prevents oxidative tissue damage and can recruit immune cells to localised areas of inflammation (Uhlar and Whitehead, 1999). Serum amyloid A is immunomodulatory, inhibiting pyrexia and down regulating pro-inflammatory events during an APR (Shainkin-Kestenbaum *et al.*, 1991; Uhlar and Whitehead, 1999).

4.1.1.2 Serum amyloid A and the acute phase in chickens

In both mammals and birds persistently elevated serum SAA levels, together with ongoing inflammatory and/or infectious disease may result in the formation of AA amyloid protein. The accumulation of this proteinaceous SAA derivative in organs and tissues gives rise to the disease AA amyloidosis (Röcken and Shakespeare, 2002). AA amyloidosis in chickens and other Galliform species was rare until Landman *et al.*, (1994) reported a new syndrome of avian amyloidosis in a flock of heavy breed brown layer chickens. Birds showed signs consistent with chronic arthritis, with inflammatory lesions in the joints and tendon sheaths. These lesions were found to be amyloid giving rise to a new arthropathic classification of amyloidosis in birds, of which Galliformes are predisposed (Landman, 1998). Serum amyloid A, when investigated in amyloidotic birds was found to be significantly higher than in healthy controls (Alasonyalilar *et al.*, 2006).

Nazifi *et al.*, (2010, 2011) found significant increases in SAA in chickens infected with IBDV and IBV. These studies show only mild 1.5- 2 fold increases in plasma SAA concentration in response to the viral infections. Similarly Seifi *et al.*, (2014) found IBV Serotype 4/91 to increase SAA serum concentrations 2-fold to 2.85±0.21 mg/L. By 5 days post inoculation, average serum concentration had returned to a level lower than the initial baseline concentration. Earlier work by Upragarin, (2005) also found SAA to increase as a result of turpentine and *S. aureus* injection with SAA levels increasing from undetectable levels pre-injection to increases of 100-1000 fold 12 hours post injection. At 72 hours post injection the mean SAA concentration of injected birds was 77.23 mg/L. Alasonyalilar *et al.*, (2006) found severely amyloidotic birds to have mean serum SAA concentration of 42.53 mg/L. However in this latter study control birds had serum concentrations of 8.48 mg/L, much higher than control levels reported in Nazifi *et al.*, (2010, 2011) and Upragarin (2005).

4.1.1.3 Quantification of serum amyloid A in chickens

The study by Upragarin (2005) is the only one to use a specific chicken SAA antibody ELISA, the other studies use a Murine antibody. Previous work undertaken but not described in this chapter found a number of anti-SAA antibodies to show no cross reactivity with chicken acute phase serum. Rabbit anti-recombinant bovine SAA, rabbit anti-pig SAA, rabbit α - bovine SAA and sheep α - bovine SAA peptides all showed no cross reactivity.

4.1.2 C-Reactive Protein

C-reactive protein (CRP) is a member of the phylogenetically ancient and highly conserved 'pentraxin' family of proteins, which also includes serum amyloid P component, a constituent of all amyloid deposits (Volanakis, 2001). This APP has been defined in humans as an exquisitely sensitive marker of inflammation and tissue damage. C-reactive protein is a major APP in humans, pigs and dogs and is the most frequently measured APP in veterinary species (Eckersall and Bell, 2010).

4.1.2.1 C-reactive protein structure and function

C-reactive protein consists of five non-covalently associated protomers arranged symmetrically around a central pore (Volanakis, 2001). Canine CRP has a

molecular weight of 100kDa, with each of the five each protomers having a molecular weight of 20kDa, human CRP contains five 25kDa protomers. Examined by electron microscopy, canine CRP resembles human CRP, the main difference between the proteins is that two of the five subunits of canine CRP are glycosylated (Ceron *et al*., 2005) giving rise to two distinct bands at 25-27 kDa when examined by 1DE.

Originally named for its ability to bind C-polysaccharide of *Streptococcus pneumonia* (Tillett & Francis, 1930), CRP has calcium ion dependent binding specificity for phosphocholine a constituent of many bacterial and fungal polysaccharides and most biological cell membranes including C- polysaccharide the teichoic acid of *Streptococcus pneumoniae* (Volanakis & Kaplan 1971). C-reactive protein binds, in a calcium-dependent manner, a diverse array of ligands including phosphatidylcholine in membrane bilayers, fibronectin, laminin, chromatin, histones, and small nuclear ribonucleoprotein, implying a role in the clearance of cellular and nuclear debris (Kravitz *et al*., 2005). On binding to the surface of apoptotic cells there is amplification of the classical pathway of complement activation, reduced terminal complement component assembly, increased phagocytosis by macrophages and sustained production of transforming growth factor-ß (Volanakis, 2001). As well as contributing to restoration of normal structure and function of injured tissues CRP's ability to recognize pathogens and to mediate their elimination by recruiting the complement system and phagocytic cells makes CRP an important constituent of the innate host defence (Volanakis, 2001).

4.1.2.2 C-reactive protein and the acute phase response in chickens

Patterson and Mora (1964) purified CRP from chicken serum and developed an assay to detect the presence or absence of CRP in chicken serum. They found CRP to be present in birds with *E. coli, Pasturella (P.) multocida* and *Staphylococcus (S.) aureus* infection, as well as Histomoniasis and adjuvant injection. The authors also found CRP positive birds in a clinically normal population. These positive birds, on post mortem, had lesions consistent with chronic respiratory disease, highlighting the use of CRP as a potential biomarker for non-clinical disease. Whether CRP is a major APP in chickens as it is in humans and other veterinary species is yet to be established. Patterson and Mora

(1965) found that CRP did not rise in chickens as quickly as it is in humans, CRP was detectable 36 – 48 hours post infection in chickens, compared to 16 -18 hours in humans. It was also noted that the CRP concentration, though not quantitated, appeared lower in chickens than in comparative human samples.

Recent studies have found the percentage of CRP positive birds to increase during heat stress (Sohail *et al.*, 2010) and experimental infection with IBV (Seifi *et al.*, 2014), *Salmonella typhimurium* lipopolysaccharide administration (Rauber *et al.*, 2014) and Fumonisin toxicity (Rauber *et al.*, 2013). Basal (pre-innoculation) concentration of CRP were reported to be 1.3±0.014 mg/L increasing significantly to 1.65±0.011 mg/L two days post inoculation with IBV (Seifi *et al.*, 2014).

4.1.2.3 Quantification of the C-reactive protein in chickens

Since the early work by (Patterson & Mora, 1964, 1965) no detailed investigation of CRP in chickens has been published. Recent studies have measured CRP in chickens using human detection kits (Sohail *et al.*, 2010) or commercial ELISA kits (Rauber *et al.*, 2013; Rauber *et al.*, 2014; Seifi *et al.*, 2014) described as being suitable for use in chickens, containing CRP-specific antibodies (USCNK – ELISA kit for C-Reactive Protein, 2016).

C-reactive protein, having a known affinity for phosphorylcholine, a constituent of bacterial and fungal polysaccharides, can be separated, concentrated and purified using a phosphorylcholine affinity column on a fast protein liquid chromatography (FPLC) system. The CRP in acute phase serum will bind the phosphorylcholine in a calcium dependant fashion. The bound CRP can be eluted from the column using a ethylenediaminetetraacetic acid (EDTA) containing buffer, the EDTA binding the calcium ions preferentially, causing the CRP to disassociate from the column.

4.1.3 Alpha-1-Acid Glycoprotein

α1 – acid glycoprotein (AGP) is a constitutively expressed, highly glycosylated protein that is found mainly in blood in varying concentrations depending on the species (Ceciliani & Pocacqua, 2007; Murata *et al.*, 2004). As well as increasing in concentration during inflammation, AGP also undergoes structural modifications of its oligosaccharide moiety, resulting in a change of both the

degree of branching and fucosylation, with the last phase of acute inflammation associated with a decrease in AGP branching to healthy levels while the plasma concentration of the protein remains at high levels indicating that pathways for the expression of the protein and for the regulation of the modifications of the protein carbohydrate moiety differ (Ceciliani & Pocacqua, 2007).

Alpha–1–acid glycoprotein protein is synthesised and secreted mainly by hepatocytes, though extrahepatic synthesis of AGP has been demonstrated in mammalian species. This local production of AGP is thought to contribute to maintenance of homeostasis by reducing tissue damage associated with the inflammatory process (Murata, *et al*. 2004). Alpha-1-acid glycoprotein has been extensively studied in humans as it is one of the most important binding proteins in plasma alongside albumin and is heavily involved in drug binding. As well as mouse and rat models, AGP has also been well documented in the domestic cat in which it is the major APP (Ceciliani & Pocacqua, 2007). In other veterinary species it has been less well documented but it does appear to have a significant role in the early stages of inflammation and infection in chickens and is described as moderate APP (O'Reilly & Eckersall, 2014).

4.1.3.1 Alpha-1-acid glycoprotein structure and function

The very unusual chemical properties of AGP are summarized by its name: it migrates with the α-1 protein group in plasma protein electrophoresis, has a low pI (2.8-3.8) and a very high carbohydrate content (> 40%). The three dimensional structure of AGP clearly resembles the conformation of a transport protein, as it is rich in ß-sheets. It has been demonstrated that AGP can bind, in non-pathological circumstances, more than 300 different molecules and drugs (Ceciliani & Pocacqua, 2007). Alpha-1-acid glycoprotein is a natural anti-inflammatory agent inhibiting neutrophil activation and increasing the secretion of IL-1 receptor antagonists by macrophages and it also helps with the clearance of LPS by binding directly and neutralising its toxicity (Murata, *et al*. 2004). This APP immunomodulates the inflammatory response, and, at the same time, act as a plasma transport protein (Ceciliani & Pocacqua, 2007)

Human AGP is a 37 kDa single polypeptide with 183 amino acids and an isoelectric point of 2.7 (Ceciliani & Pocacqua, 2007). In contrast chicken AGP is 22.3kD has a isoelectric point of 5.11 and contains 203 amino acids

(UniProt.org). Structurally the AGP protein in chickens, like mammals, is heterogeneous in its glycosylation differing in its reactivity to concanavalin (ConA), giving rise to a Con-A reactive and Con-A unreactive forms of AGP. Inoue, *et al*. 1997 investigated the changes in heterogeneity of chicken AGP in response to in experimental infection with IBDV. In control chickens there was 47% Con-A-unreactive and 63% ConA-reactive AGP. This changed as a consequence of infection especially with inoculation of the highly virulent IBDV strain, where Con-A reactive increased to 80% in the early stages of infection. Infection with LPS resulted in an increase of 87% the Con-A reactive AGP. The biological effects of AGP are dependent upon its glycosylation type, with Con-A unreactive AGP exhibiting a stronger inhibitory effect of lymphocyte proliferation and mixed lymphocyte proliferation than the Con-A reactive. Also the inhibitory effects of AGP on neutrophil superoxide anion generation depends on the glycan structure with Con-A unreactive being less inhibitory that the Con-A reactive (Inoue, *et al*. 1997).

4.1.3.2 Alpha-1-acid glycoprotein and the acute phase in chickens

A large number of studies have investigated AGP in chickens in response to both bacterial and viral infection. Following a single *Escherichia (E.) coli* LPS injection AGP plasma concentrations increased significantly 12 hours post injection with serum levels increasing 4 fold to peak 24 hours post injection (Takahashi *et al.*, 1998). Nakamura *et al.,* (1998) found infection of *E. coli* LPS resulted in an increase in AGP that peaked 48 hours post infection with a return to normal levels over 7 to 14 days. Repeated injections of *E. coli* LPS was found to lower the responses of plasma AGP concentrations (Takahashi *et al.,* 1995) while repeated injections of LPS at different concentrations resulted in different AGP profiles, with the higher concentration of LPS giving higher AGP values (Takahashi *et al.,* 1998). Age and sex do not significantly alter AGP concentrations (Takahashi *et al.,* 1995). Adler *et al.,* (2001) using *Salmonella* serotype Typhimurium LPS and turpentine to illicit an APR found AGP levels increased 4 fold, peaking 24 hours post infection before returning to normal levels 6 days post infection.

A number of studies have investigated AGP during viral infections both with and without vaccination. Experimental infection with infectious bronchitis (IB) and

infectious larygotracheitis virus (ILTV) increased AGP serum concentrations, with AGP higher in the IB than the ILTV infected birds (Nakamura *et al.*, 1996). Inoue *et al.*, (1997) found significant increases in serum AGP as a result of infection with a highly virulent infectious bursal disease virus (IBDV) strain and a virulent IBDV reference strain. The highly virulent strain increased AGP concentrations six fold and the virulent reference strain three fold. The chickens infected with the highly virulent strains also had extensive lesions in addition to the severe bursal lesions. Chickens receiving the attenuated vaccine strain of IBDV showed only a slight increase in AGP and no lesions in the bursa or liver (Inoue *et al.*, 1997). Chickens challenged with low pathogenic avian influenza (LPAI) showed increases in AGP up to 48 post infection; however those that received LPAI vaccine were shown to have significantly lower AGP concentrations than unvaccinated controls (Sylte and Suarez, 2012).

As a result AGP measurement has been used in a wide variety of poultry research areas most notably in the area of nutrition where experimental diets are followed by an immunogen challenge usually in the form of a bacterial LPS and the AGP concentration determined. The changes in AGP concentrations as a result of nutritional changes have been particularly well documented by Takahashi *et al.* (1995; 2002; 2009). The use of APPs as a physiological marker for overall health and welfare is another potential use of APP measurement. In comparing different egg laying systems Salamano *et al.*, (2010) compared the serum concentrations of AGP and albumin. Mean AGP serum concentrations, when measured at 2 weeks and 2 months post arrival at accommodation, were higher in hens kept in conventional and modified cages than hens in a free range system. However after 4 months the free range hens had higher mean AGP than both modified and conventionally caged groups, with the mean AGP of the free range hens having increased significantly from 2 months previously. AGP was used to evaluate stress and immunity of organically produced broilers compared to traditionally produced broilers. The organic broilers had serum AGP concentrations that were significantly higher (P=<0.001) than the conventionally produced broilers, this was attributed to restriction of prophylactic medicines coupled with access to the outside (Tuyttens *et al.*, 2008).

4.1.3.3 Quantification of alpha-1-acid glycoprotein in chickens

α1 – acid glycoprotein is one of the most widely measured APPs in chickens owing to the commercial availability of easy to use and chicken specific AGP kits (ECOS Institute, Miayagi, Japan). This kit contains anti-chicken α-1-acid glycoprotein antibodies of rabbit origin and commercial single RID test kits are available for measurement of AGP in chickens (Takahashi et al. 1994). Other immunoassay kits measuring AGP in chickens are now available.

4.1.4 Apolipoprotein AI

Plasma lipoproteins are water-soluble macromolecules composed of lipids and one or more specific proteins called apolipoproteins (apos) (Franceschini, 2003). There are several classes of lipoproteins: high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons, classified on the basis of its floatation density or size (Tan *et al.*, 2005). Avian HDL comprise more than 80% of plasma lipoproteins and their lipid and protein composition very closely resemble that of human HDL (Lamon-Fava *et al.*, 1992). Apolipoprotein AI (Apo-AI) is the major protein fraction of HDL in both birds and mammals (Kravitz *et al.*, 2005).

The pattern of tissue expression of the Apo-AI gene in birds is however very different from mammals in which Apo-AI synthesis is restricted to liver and intestine (Lamon-Fava *et al.*, 1992). In chickens, Apo-AI synthesis occurs in a number of peripheral tissues in addition to the liver and intestine. In this respect, chicken Apo-AI closely resembles mammalian apolipoprotein E (ApoE), which is expressed at high levels in brain, kidney, lung, and other peripheral tissues. Since ApoE has not been detected in the plasma lipoproteins of chickens it is suggested that Apo-AI is the functional homologue of mammalian extrahepatic ApoE (Rajavashisth *et al.*, 1987; Lamon-Fava *et al.*, 1992). Comparing the total Apo-AI mRNA in the peripheral tissues, liver and intestine of chickens reveals that the peripheral tissues do not contribute a major portion to the circulating plasma Apo-AI functioning instead in cellular cholesterol metabolism or local lipid transport. On a cell basis, Apo-AI is most abundant in the intestine, whereas on a whole organ basis, the content of Apo-AI mRNA is very similar in liver and intestine, suggesting that both organs contribute almost equally to the whole body Apo-AI synthesis (Rajavashisth *et al.*, 1987).

4.1.4.1 Apolipoprotein AI structure and function

A prominent structural feature of apolipoproteins is an amphipathic α-helix in which the hydrophobic and hydrophilic faces are separated by positively charged residues, providing a hydrophobic side for interaction with lipids and a hydrophilic surface to interact with physiological fluids (Rajavashisth *et al.*, 1987; Franceschini, 2003). Chicken Apo-AI contains 264 amino acid and has a molecular weight of 30.6 kDa and an isoelectric point of 5.59 (Uniprot.org/P08250) and shows 49% and 42% homology to human and rat Apo-AI respectfully (Rajavashisth *et al.*, 1987).

In mammals Apo-AI is an antioxidant incorporating esterified cholesterol into HDL and stabilising paraoxonase 1 and preventing lipid peroxidation (Kravitz *et al.*, 2005). The cardioprotective role of high levels of HDL has been well described in humans with a strong inverse correlation between plasma HDL levels and coronary heart disease, and Apo-AI seems to the major factor in the anti-atherogenic activity of HDL because of the multiple roles it plays in reverse cholesterol transport (Franceschini, 2003; Kravitz *et al.*, 2005). Reverse cholesterol transport is the process whereby excess cholesterol in peripheral tissues including the arterial wall is esterified in the plasma and transported to the liver for excretion (Kravitz *et al.*, 2005). In birds, HDLs are responsible for the redistribution of cholesterol, cholesterol esters, and other nonpolar lipids from peripheral tissues to the liver for reuse or excretion in the bile (Roman *et al.*, 2009).

4.1.4.2 Apolipoprotein AI and the acute phase in chickens

In mammals Apo-AI is a negative acute-phase protein decreasing by up to 25% during acute inflammation. Pro-inflammatory mediators IL-6, IL-1ß, and TNF-α that usually stimulate the synthesis of positive APPs such as CRP and SAA, inhibit the synthesis of Apo-AI (Kravitz *et al.*, 2005). In pigs Apo-AI is a negative APP, where it decreases in experimentally induced acute inflammation (Carpintero *et al.*, 2005), African swine fever, Aujeszky's disease (Carpintero *et al.*, 2007) and during road transport (Piñeiro et al., 2007). It also appears to reduce during the APR in cows infected with *Salmonella* Typhimurium (Oikawa *et al.*, 1997).

As a lipoprotein expressed widely in peripheral tissues chickens Apo-AI is regularly identified in both the serum and tissue proteomes of chicken (O'Reilly,

2013). However its behaviour during an APR and possible use as a biomarker for infection and inflammation in chickens has not yet been determined. Apolipoprotein AI has been measured in a study on the effect of feeding regime on hepatic lipid metabolism in sexually mature broiler breeders, where it was found that the expression Apo-AI was significantly higher in birds reared using a SKP feeding regime compared to those reared on a everyday feeding regime (Ekmay *et al.*, 2010).

4.1.4.3 Quantification of apolipoprotein AI in chickens

Kits for measuring chicken Apo-AI are widely available and use immunoassay methods with chicken specific antibodies. Despite this there are no reports in the literature of Apo-AI being measured in chickens. A mutant chicken (WHAM) identified and used as a model for spontaneous HDL deficiency has over a 90% reduction of HDL and Apo-AI compared to normal chickens. The serum concentrations of Apo-AI in the WHAM and normal chickens were 0.08±0.006 g/L and 0.95±0.21 g/L respectfully (Poernama *et al.*, 1992). This normal Apo-AI concentration is lower than that reported for pigs, where the mean concentrations ranged from 2.3–3.08 g/L in pre-challenged groups in the series of studies by Carpintero *et al.*, (2005).

4.1.5 Transthyretin

Thyroid hormones (THs), lipophilic hormones that modulate growth and development, are bound to specific carrier proteins in plasma to ensure adequate distribution to target tissues (Yamauchi & Ishihara, 2009). Extracellular TH are distributed by thyroxine-binding globulin (TBG), transthyretin (Ttn) and albumin (Eguchi *et al.*, 2008; Yamauchi & Ishihara, 2009). In humans, 70–80% of THs in plasma are bound to TBG whereas in chickens, most THs are bound to Ttn and albumin as TBG is not synthesized in avian species (Eguchi *et al.*, 2008).

Hepatically synthesised Ttn is secreted alongside TBG and albumin into plasma. Transthyretin is also synthesised in the choroid plexus of the brain and secreted into the cerebrospinal fluid (CSF) of adult mammals, birds and reptiles (Yamauchi & Ishihara, 2009). In rats, Ttn constitutes 50% of the total protein secreted by the choroid plexus, with all Ttn made by choroid plexus secreted towards the brain (Chang *et al.*, 1999). In chickens Ttn from circulation

accumulates in the yolk during oogenesis and is expressed embryonically before the vascular system and hypothalamic– pituitary–thyroid axis are established, where it plays a role in the transport and distribution of THs from the yolk to specific embryonic sites (Yamauchi & Ishihara, 2009). In adult chickens Ttn gene expression has been detected, by RT-PCR, in the kidney, lung, spleen and intestine (Yamauchi & Ishihara, 2009).

4.1.5.1 Transthyretin structure and function

Transthyretin has been detected in the plasma of fetal and adult eutherians, herbivorous (Australian) marsupials and some omnivorous (American) marsupials and birds (Chang *et al.*, 1999; Yamauchi & Ishihara, 2009). It has been detected only at low levels, in the plasma of adult reptiles, amphibians and fish, in which the expression of Ttn is modulated in developmentally specific and species-specific manners (Yamauchi & Ishihara, 2009); recent studies have suggested that the extrahepatic expression of Ttn is more predominant in lower vertebrates than in higher vertebrates (Yamauchi & Ishihara, 2009). Transthyretin is one of the most strongly conserved plasma proteins as sequences from mammals show 87 % sequence identity between species. Chicken and lizard Ttn show 75% and 66% sequence similarity with the human protein, respectively (Sunde *et al.*, 1996). The sequence of amino acids in the thyroid hormone binding site regions of Ttn is totally conserved between species (Chang *et al.*, 1999). Each molecule of Ttn is composed of four identical subunits (each composed of 127 amino acids) which coalesce non-covalently to generate a non-glycosylated edifice with a molecular mass of 55 kDa and a central channel with two potential binding sites for the thyroid hormones (Chang *et al.*, 1999; Ingenbleek & Bernstein, 2015).

Transthyretin is described as being a negative acute phase reactant, decreasing in concentration in the plasma during an APR (Gruys *et al.*, 2005). The decrease in plasma Ttn alongside cortisol-binding globulin and retinol-binding protein during an APR indicates an increased availability of the hormones bound to these proteins. The negative APPs are therefore described by some authors as ‘acute booster reactants’ (Ingenbleek & Young, 1994). Used as a biomarker in human medicine Ttn has received strong support for assessing a broad array of diseases comprising metabolic and septic disorders though some researchers in the human

field have cast doubt on the clinical reliability of Ttn (Ingenbleek & Bernstein, 2015). The exquisitely sensitive response of Ttn to the restriction of dietary amino acid supply, attributed to its small pool size, its short half life, and its high content of tryptophan (the narrowest of all indispensable amino acid pools in mammalian tissues) also make Ttn a biomarker of nutritional status and energy and protein adequacy (Ingenbleek & Bernstein, 2015).

4.1.5.2 Transthyretin and the acute phase response in chickens

As an acute phase reactant Ttn has not been studied in chickens and no information as to its behaviour or measurement during APR has been published. Transthyretin is a negative APP in pigs, where serum concentration of Ttn was shown to decrease rapidly and stay depressed during *Streptococcus suis* and *Mycoplasma hyosynoviae* infections (Campbell *et al.*, 2005; Heegaard *et al.*, 2011).

4.1.5.3 Quantification of transthyretin in chickens

In pigs Ttn has been quantified using an in-house ELISA that utilised an anti-human Ttn antibody (Campbell *et al.*, 2005; Diack *et al.*, 2011; Heegaard *et al.*, 2011). Reference ranges are reported as 174–610 mg/L in pigs (Diack *et al.*, 2011), and 300–330 mg/L and 250–270 mg/L in male and female humans (Ingenbleek & Bernstein, 2015).

4.1.6 Haemopexin

Haemopexin (Hpx) is a haem binding protein synthesised mainly by hepatocytes and has been detected in the plasma of all mammals to date and is an established APP in chickens and other birds. In mammals Hpx is synthesized mainly in the liver though extrahepatic synthesises has been demonstrated in skeletal muscle, retina, nervous system and kidney (Grieninger *et al.*, 1986; Adler *et al.*, 2001; Tolosano & Altruda, 2002; Wicher & Fries, 2010).

4.1.6.1 Haemopexin structure and function

Haemopexin has the highest affinity for iron of any known haem-binding protein and is important in the clearance of haem following haemorrhage, trauma, or infection (Ascenzi *et al.*, 2005). During homeostatsis low concentrations of haem in the plasma occurs due to oxidation of haemoglobin, which is released during the enucleation of erythroblasts. This haemoglobin dissociates into αß dimers

which are rapidly bound by haptoglobin (Hp) and metabolised. Plasma haemoglobin (Hb) that remains unbound to Hp is quickly oxidized into ferrihaemoglobin, before dissociation into globin and ferrihaem. The haem is first bound by albumin then transferred to Hpx for which it has a much higher affinity. Haemopexin binds to the non-protein pyrrole rings within the haem molecule and once bound undergoes a conformational change allowing for interaction with a specific Hpx receptor, mainly expressed on the hepatocyte membrane. It is internalised and the haem is catabolised (Tolosano & Altruda, 2002; Tolosano *et al*., 2010). Haemopexin can then be released intact into the bloodstream and the haem is degraded and the iron is reutilised (Ascenzi, *et al*. 2005). Receptors for haem–Hpx are expressed by liver parenchymal cells as well as retinal pigment epithelia cells, lymphocytes, and several cell lines (Tolosano & Altruda, 2002).

Haemopexin is a multifunctional protein having involvement in iron homeostasis, antioxidant production and signalling pathways that promote cell survival and gene expression (Tolosano *et al.,* 2010). The predominant function of Hpx however is to sequester and transport haem, preventing both haem catalysed oxidative damage and haem- bound iron loss (Tolosano & Altruda, 2002). In addition to its role during homeostasis, Hpx alongside Hp is also important for the clearance of haem following haemorrhage, trauma, infection or other haemolytic crisis (Ascenzi *et al*., 2005; Tolosano *et al*., 2010). During such times the presence of Hpx prevents haem-catalyzed oxidative damage, the pro-inflammatory effects of free haem and limits the growth of many pathogens by preventing them from acquiring this readily available source of iron (Buyse *et al*., 2007)

The structure of chicken Hpx differs significantly from mammalian Hpx being a larger, 52kDa protein with a different glycosylation pattern: possessing α1-glycoprotein as oppose to a ß1-glycoprotein seen in mammals. In keeping with the finding of a simpler carbohydrate structure, chicken Hpx exhibits a single band on 1DE under both non-denaturing and denaturing conditions whereas mammalian Hpx shows several bands. In contrast, the isoelectric focusing pattern of chicken haemopexin is very complex, revealing at least nine bands between pH 4.0 and pH 5.0 (Goldfarb *et al*., 1986).

4.1.6.2 Haemopexin and the acute phase response in chickens

Barnes *et al.,* (2002), using *E. coli* LPS to induce an APR found a 2.6 fold increase in Hpx. Adler *et al.*, (2001) used *Salmonella* Typhymurium LPS and complete Freund's adjuvant and found Hpx levels to increase three fold 24 hours post infection, Hpx levels were still significantly higher than the control birds 14 days after the LPS challenge. Garcia *et al,.* (2009) found Hpx to increase by approximately 1.5 fold in response to intra crop *Salmonella enterica* serovar Gallinarum, with serum concentration decreasing from 7 days post infection.

L-Carnitine, synthesized *in vivo* from methionine and lysine, is known to have immunomodulary properties. Buyse *et al.*, (2007) found that while plasma Hpx (and AGP) were significantly increased in birds injected with LPS, the increase was more pronounced in the L-carnitine supplemented chickens, suggesting that extra L-carnitine in the diet of broiler chickens can enhance or advance the APR. In a similar study supplementation of *Astragalus* polysaccharide was found to attenuate the increase in Hpx (and AGP) induced by LPS challenge in chickens (Liu *et al.*, 2015).

4.1.6.3 Quantification of haemopexin in chickens

Purification of Hpx from chicken serum is possible using a haem affinity chromatography. Purified chicken Hpx was used to raise a chicken specific antibody to Hpx which was used in rocket immunoelectrophoresis to determine Hpx concentrations (Adler *et al.*, 2001; Buyse *et al.*, 2007). Using this method a basal concentration of 0.15 g/L was reported by Buyse et al., (2007) which is lower than the basal concentration in humans of 0.5–1.2 g/l humans (Tolosano & Altruda, 2002). The administration of LPS resulted in Hpx concentrations increasing to approximately 0.3 – 0.6 g/L (Buyse *et al.*, 2007).

4.1.7 Quantitative Proteomics

MS(MS) based quantitative proteomic approaches are well-suited to the discovery of protein mediators and biomarkers of disease (Cretu *et al.*, 2015) which together with the introduction of new technologies in MS allows for the identification and quantification of thousands of proteins in a single experiment through different sets of approaches called shotgun proteomics (Wilson, 2013).

Shotgun proteomics, which is named for its analogy with shotgun genomics is conceptually accepted as the use of enzymes, usually trypsin, to digest proteins and characterise the proteins in the mixture by tandem MS, this is termed the bottom up approach. The top down approach is the characterisation of intact proteins, this approach is better for the determination of post translational modifications and the characterisation of isoforms, though there are disadvantages to this approach and methods are still under development. The middle down approach is the intermediate approach of using partial or limited proteolytic digests for protein identification (Nogueria & Domont, 2014).

Descriptive proteomics, the identification of proteins, gives extremely important qualitative information on biological systems, however it is quantitative information on protein abundance that provides precise and richer results (Nogueria & Domont, 2014). Two main approaches are used in quantitative MS based proteomics: stable isotope labelling and label-free quantification (Arike & Peil, 2014) which is absolute quantification where the protein concentration or copy number is determined, or relative quantification respectfully (Nogueria & Domont, 2014). Quantification with stable isotopes is based on the mass difference between labelled and unlabelled ions in MS analysis. Proteins are chemically, metabolically, or enzymatically labelled with molecules that have a combination of light and heavy isotopes of ^{15} N, ^{13} C, ^{18} O, and ^{2} H in their composition. After mixing, the peptides labelled with different isotopes are identified as pairs in the MS spectra or as reporter ions in the MS/MS and the intensity ratio between the isotope variants reflects the fold-difference between their abundances (Arike & Peil, 2014; Nogueria & Domont, 2014).

For label-free quantification there are two basic approaches the first is by counting the number of spectra acquired for a given protein in a given sample. This method is termed spectral counting (Salvidor & Levin, 2014) and it is the most simple label-free quantification technique based on the observation that the more abundant the protein is the more peptides can be identified from it (Arike & Peil, 2014). Spectral counting implies a counting and a comparison of the number of fragment-ion spectra (MS/MS) acquired for peptides of a given protein. Due to the empirical observation that the number of tandem mass spectra of a particular peptide increases with an increasing amount of the

corresponding protein, a relative quantification of proteins between different samples is possible (Megger *et al.*, 2013). The second method uses the measurement of chromatographic peak areas (also termed MS signal intensities) of peptide precursor ion intensity and this has been shown to produce a more accurate quantification (Salvidor & Levin, 2014). The quantification is based on the differential peak intensity of the peptides in each MS scan (Cretu *et al.*, 2015). Depending on the chromatographic method (e.g. reversed-phase liquid chromatography) the peptides are separated according to their particular physical properties (e.g. hydrophobicity, charge), subsequently ionized in an ion source and finally detected in a mass spectrometer. In the acquired mass spectrum each peptide of a particular charge and mass generates one monoisotopic mass peak. The intensity of this peak as a function of the retention time can be visualized in an extracted ion chromatogram (XIC) and the area under the curve (AUC) can be determined. The areas of chromatographic peaks have been shown to correlate linearly in a wide range with the protein abundance which makes their measurement feasible for quantitative studies (Megger *et al.*, 2013).

Label-free proteomics has emerged as a high-throughput method for quantitative clinical proteomics studies (Megger *et al.*, 2013) as such experiments often result in tens to hundreds of candidate biomarkers, therefore, in the context of biomarker discovery, high-throughput MS-based proteomics is a powerful tool for obtaining disease-specific proteome profiles of biological materials (Cretu *et al.*, 2015). In the human field of biomarker identification, plasma is known to represent a diverse proteome and while an excellent source of potential disease markers, as proteins secreted by tissues are diluted in blood, and are often undetectable by current MS methods (Cretu *et al.*, 2015). To aid protein identification samples can be pre-fractionated (e.g. via 1D-PAGE, isoelectric focussing, ion exchange chromatography, high-pH reversed-phase liquid chromatography) prior to reverse phase liquid chromatography and each fraction can then be analyzed in a single LC–MS/MS experiment. A clear advantage of such a 2D setup is a decomplexation of the sample leading to a higher proteome coverage as well as the possibility to select particular fractions of interest for the subsequent analysis (Megger *et al.*, 2013). Depending on the MS used for a label-free analysis, different acquisition modes can be used, namely a data-

dependent acquisition (DDA) and a data-independent acquisition (DIA). The former one includes the acquisition of a survey scan and a subsequent fragmentation of selected precursor peptide ions (Megger *et al.*, 2013).

4.1.7.1 Planning a quantitative proteomic study: pre-fractionation and sample preparation

While shotgun proteomics has become the method of choice for identifying and quantifying proteins in large scale studies because of high data throughput and better protein detection sensitivity (Nesvizhskii, 2007), digesting all the proteins within a biological sample such as plasma into peptides will result in a very complex sample (Hu *et al.*, 2007; Nesvizhskii, 2007). As a result the amount of mass spectra generated from shotgun analysis of complex samples such as serum is very difficult to interpret because of the large number of peptides generated from trypsin digestion (Fournier *et al.*, 2007), the complexity of which is much higher than simply the sum of all the tryptic peptides from a mixed protein sample, as miscleavages and truncations occur during digestion giving rise to much higher than predicted numbers of peptides (Westermeier *et al.*, 2008). The tens of thousands of peptides generated from a tryptic digestion of a sample will have a wide dynamic range of concentrations. These peptides may have similar m/z ratios and different levels of abundance, creating a challenge for the MS with respect to the detection and identification of low abundant proteins. Therefore powerful separation techniques are required to maximise the number of peptides for analysis for MS (Fournier *et al.*, 2007).

Within a complex protein sample such as serum or plasma the concentrations of proteins are not evenly distributed and it is very difficult for MS to detect and analyse very low abundant proteins. The protein concentrations of plasma span over ten orders of magnitude (Westermeier *et al.*, 2008) which together with the generation of the tens of thousands of peptides mean that identifying a low abundant protein within a serum sample can be very difficult. To increase the chance of identifying peptides from the target proteins, enriching the source material to increase the concentration of a low abundant protein is often necessary. For serum this can simply be achieved by removing the high abundant proteins. In plasma approximately 90% of the proteins are albumin and IgG and just twenty-five high abundant proteins represent 99% of the total protein

content, however the remaining proteins are still spread over a huge concentration range (Westermeier *et al.*, 2008). Other enrichment methods will target the proteome of particular cellular locations or organelles.

The initial part of this study is concentrated on identifying the APPs SAA and CRP in chicken serum with a view of identifying suitably tryptic peptides from these APPs for quantification purposes. The molecular weights of SAA and CRP are 14.1 kDa and 25.6 kDa respectfully, making these suitable candidates for enrichment by molecular weight fractionation prior to trypsin digestion. By fractioning based on molecular weight many of the high abundant proteins such as albumin and IgG will be excluded. Utilising CRP's strong affinity for phosphorylcholine an affinity column on a fast protein liquid chromatography (FPLC) system can separate and concentrate CRP from acute phase serum. This can be used as a further enrichment method for CRP.

The shotgun proteomics method for this study is based on the coupling of high performance liquid chromatography (HPLC) and MS. The digested peptides from both whole serum and the enrichment samples will be analysed by multidimensional chromatography coupled to tandem MS/MS. Multidimensional separation can be performed over two independent separation techniques that can include ion exchange, size exclusion and reverse phase affinity (Fournier *et al.*, 2007). This study will utilise reverse phase C18 chromatography for both dimensions, with high pH (pH10) and low pH (pH3) for the 1st and 2nd dimensions respectfully. The peptides are then sequenced using tandem MS (MS/MS) and automated database searching is then used to identify the proteins (Lange *et al.*, 2008). This study will utilise the technologies of the Q-Exactive Hybrid Quadrupole–Orbitrap mass spectrometer (Thermo Scientific) which allows both the targeted and untargeted screening (Thermo Scientific).

The first stage of this study will utilise untargeted screening to identify all the proteins present in the whole serum of three acute phase pools and the enriched samples, using the peak area the MS/MS spectra which uses the area under the curve (AUC) to give a relative quantification of each protein following normalisation using intensity.

When selecting peptides for each protein target it is critical that peptides are both detectable and unique to the protein of interest (proteotypic) and also

accurately represent the level of the protein (quantotypic) (Worboys *et al.*, 2014). Peptides that are quantotypic are readily ionized and observed in the mass spectrometer with no signal loss due to miscleavage or post translational modification. The proteolytic digestion of proteome proteins is frequently incomplete, and miscleavages are often generated in addition to or instead of the limit peptides produced. The goal of quantitative proteomic experiments is to quantify the proteins, not their peptides *per se*, so quantotypic peptides should be stoichiometric with the parent protein to enable accurate quantitation (Lawless & Hubbard, 2012). Several factors may impact the quantotypic properties of peptides such as differential post translational modification, alternative splicing and the completeness of proteolytic digestion. Selection of optimal quantotypic peptides is crucial to ensure accurate quantification of protein levels (Worboys *et al.*, 2014). Once peptides of the targeted proteins have been identified and validated the Q-Exactive will be used with the targeted mode (single ion monitoring SIM/targeted MS/MS) screening to identify and quantify only the peptides of interest. The basic concept is to monitor the presence and intensity of specific transitions consisting of pairs of precursor ion m/z and its product ion m/z. Single ion monitoring (SIM) and targeted MS/MS analysis provide high sensitivity and high specificity for peptide selection since only desired peptides are selected and analysed in both MS and MS/MS mode and other signals are regarded as noise (Zhi *et al.*, 2011).

4.1.8 Aims of this study

The purpose of this study is to utilise proteomic technologies to identify and quantify APPs in chickens, using enrichment methods where necessary to aid in the identification of low abundant APPs. Proteins found to be differentially abundant proteins between acute phase and non-acute phase samples were also targeted and measured with a view of identify novel biomarkers of the APR.

Serum amyloid A was targeted as it is the only major APP described in chickens, while CRP was targeted as it is the major APP in many species, yet descriptions in chickens remain scant. Initially SAA and CRP were enriched using molecular weight fractionation and a phosphorylcholine affinity column for CRP. These enriched fractions together with whole serum from acute phase and non-acute phase samples underwent trypsin digestion and shotgun analysis using

untargeted screening with UPLC coupled to a Q-Exactive Hybrid Quadrupole–Orbitrap mass spectrometer (Thermo Scientific). Known APPs together with differentially expressed proteins were targeted using the same method, this time using the Q-Exactive Hybrid Quadrupole–Orbitrap mass spectrometer in a targeted mode (data dependant single ion monitoring SIM/targeted MS/MS) screening to identify and quantify only the targeted peptides of interest. It was the hypotheses of these studies that SAA and CRP would be identified in enriched sera and subsequently quantified. Furthermore, identifying proteins, differentially expressed between three acute phase groups would also allow the targeting of other, novel, acute phase reactive proteins as well as the quantification and characterisation of other known APPs.

4.2 Method

A diagram, detailing the experimental process and proteomic workflow is contained in Appendix 4.2.

4.2.1 Enrichment and shotgun analysis of acute phase serum

4.2.1.1 Identifying C-reactive protein in chicken serum

Using an AKTA Fast Protein Liquid Chromatography (FPLC) (GE Healthcare, Life sciences) a pooled sample of acute phase chicken serum was injected on to an immobilized p-aminophenyl phosphoryl choline gel column (Thermo Scientific 20307), following column equilibration with a binding buffer (0.1M Tris, 0.1M NaCl, 2mM $CaCl_2$ pH8). Following one hour incubation at room temperature, the column was flushed with binding buffer to remove unbound proteins. An elution buffer containing ethylenediaminetetraacetic acid (EDTA) (0.1M Tris, 0.1M NaCl, 10mM EDTA pH8) was pumped through the column and eluates collected over time in a fraction collector. Four elutions containing protein were pooled, dialysed overnight at 4˚C to and centrifuged in an Amicon centrifuge tube (Millipore Amicon Ultra centrifugal filters 0.5mL 3K UFC500324) to concentrate the protein for 1DE, which was undertaken using methods detailed in Chapter 2, Section 2.2.2.1. Western blotting using rabbit α-human CRP and sheep α-dog CRP at a 1:500 dilution, with Goat anti-rabbit IgG HRP (Abcam 6721) and Rabbit anti-sheep IgG HRP (Abcam 6747) as secondary antibodies respectfully, was undertaken using methods detailed in Chapter 2, Section 2.2.2.2. Bands were excised and the proteins identified using nanoflow HPLC electrospray tandem MS(nLC-ESI-MS/MS) detailed in Chapter 2, Section 2.2.2.3.

4.2.1.2 Sample collection

To obtain acute phase serum with potentially high concentrations of SAA and CRP, a flock of broiler chickens of 25 days of age from a commercial farm in Scotland were accessed on site for poor growth, lameness and generalised signs of ill health. Within a single house 19 birds were selected, culled and blood was recovered immediately post-mortem into serum tubes. Three serum samples were obtained from a different company from sick birds that were later diagnosed with Adenovirus infection. Serum concentrations of AGP, was

determined using a chicken specific single immunodiffusion test kit (ECOS Institute, Miyagi, Japan). The Adenovirus infected birds showed up to a 10 fold increased AGP concentration and were categorised as being highly acute phase (HAP). Acute phase (AP) samples, showing up to 5 fold increase in AGP compared to healthy chicken and samples where the AGP was within the normal ranges and non-acute phase (NAP) were pooled to create two further groups (Table 4.1).

Group	Acute phase status	n
Pool – 1	Highly acute phase (HAP)	3
Pool – 2	Acute phase (AP)	6
Pool – 3	Non- acute phase (NAP)	6

Table 4.1: The acute phase status of three groups for shotgun analysis.

4.2.1.3 Protein Quantification

Serum was diluted 1:10 and 1:5 for acute phase and healthy samples respectfully and protein concentrations determined using RC DC protein assay kit (BioRad 500-01190) as follows: Bovine serum albumin (BSA) (Thermo scientific Pierce BSA assay standards) was diluted 0 – 1.4 µg/µl, and 25µl of each used as standard. The standards and diluted samples were mixed with 125µl of RC reagent 1, vortexed and incubated for 1 minute at room temperature (RT). An equal volume of reagent 2 was added, the samples centrifuged at 15,000g for 10 minutes at 4ºC and the resultant pellet was dissolved in solution A (reagents S + A), vortexed and incubated at RT for 5 minutes. To this 1ml of reagent B was added, vortexed and incubated at RT for 15 minutes, before absorbance was measured at 750nm on a spectrophotometer. The absorbance for the standards were plotted to produce a curve from which the protein concentration of the samples was calculated.

4.2.1.4 Trypsin digestion

Three pools, as detailed in Table 4.1, containing equal quantities of protein from each sample was reduced with the addition of 10mM DTT in 50mM ammonium bicarbonate and heated in the dark at 56ºC with agitation for 40 minutes. Samples were alkylated with 20mM iodoacetamide in 50mM ammonium

bicarbonate and left at RT in the dark for 30 minutes before undergoing a second 10 minute RT reduction using 11mM DTT in 50mM ammonium bicarbonate to eliminate any excess iodoacetamide. Samples were precipitated using 2D clean up kit from GE Healthcare (80-6484-51) during which 300µl of precipitant was added to each sample, vortexed and incubated on ice for 15 minutes. The same volume of co-precipitant was added, vortexed and the tubes centrifuged at 15,000g for 15 minutes at 4ºC and the supernatant removed. To wash the pellet a further 40µl of co-precipitant was added to the undisturbed pellet, incubated on ice for 5 minutes, centrifuged at 15,000g for 5 minutes at 4ºC and the wash removed. To the pellet 25µl of H_2OmQ was added and vortexed to disturb the pellet and 1ml of -20ºC acetone and 5µl of wash additive was added, the samples vortexed and the samples incubated at -20ºC for 1 hour. The samples were centrifuged, and to the final pellet 90µl of 50mM ammonium bicarbonate was added. For the first of two trypsin digestions 1µg of trypsin in 25mM ammonium bicarbonate was added to each sample, vortexed and incubated at 37ºC overnight. The following day 0.5µg of trypsin in 25mM of ammonium bicarbonate with 80% acetonitrile was added to each sample and incubated at 37ºC for 3 hours at 600rpm. To halt the digestion 0.5% of TFA was added to the final volume to acidify the pH, inhibiting the activity of the trypsin enzyme. Samples were vacuum centrifuged and 100µl of 0.1% TFA was added to the resultant pellet. Thermo Scientific Pierce C18 tips (87782) were used as according to the manufacturers instruction on each sample to remove 3.5µg of digested peptide, which was recovered in a final elution buffer of ACN 50% TFA 0.1%. This was vacuum centrifuged and the resultant pellet was re-suspended in 3.5µl of Waters MassPREP Digestion Standard Mixtures (MPDS-mix) #1 for samples 1 and 2 and into mixture #2 for sample 3. Into this the same volume of TFA and H_2OmQ were added.

4.2.1.5 Quantitative Proteomics

Shotgun analysis was performed on each of samples using 2D nano UPLC system (RP x RP nano Acquity, Waters), coupled to a Q-Exactive hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific), in conjunction with Dr. Gabriel Mazzucchelli and Professor Edwin De Pauw at the MS Laboratory at the

University of Liege, Belgium during a short term scientific mission of the COST Action on Farm Animal Proteomics.

The peptides were added over two dimensions to the mass spectrometer using liquid chromatography. The Q-Exactive-Orbitrap Mass spectrometer (Thermo) then performed MS/MS spectra analysis, and comparisons were made to the *Gallus gallus* protein database (Uniprot) using Sequest (Thermo Scientific) and Mascot (Matrix Science).

4.2.1.6 Protein fractionation

To target SAA and CRP serum was fractionated by molecular weight to create a sample rich in low molecular weight proteins to increase the sensitivity of the shotgun analysis and identifying the target peptides. From pools 1 and 2, 500µg of protein underwent a (2D) protein clean up method as described earlier, in duplicate, so as fractionate two aliquots from each pools. The GE Healthcare 2D clean-up kit (80-6484-51) was used to precipitate the protein, using 450µl of precipitate and co-precipitate. After 1 hour at -20ºC in acetone wash buffer, the resultant pellet was re-suspended in 8µl of 1M DTT, 30 µl of x5 Tris acetate sample buffer (42302) and 112µl H_2OmQ. Vortex was used to ensure the pellet was dissolved and the samples heated at 50ºC for 10 minutes.

Protein Discovery GelFree® 8100 Fractional system (Expedeon, Cambridge, UK), utilising a 10% cartridge kit to fractionate proteins from 3.5 – 100kDa was used to fractionate proteins from pools 1 and 2. An initial fractionation examining the protein concentrations of 12 fractions collected using the recommended protocol revealed that the first five fractions to be very low in protein. Because the SAA protein would fractionate within these initial fractions, the programme was extended as detailed in Table 4.2 to separate the early fractions further.

Step	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Voltage	50	50	50	50	50	50	100	100	100	100	100	100	100
Time	16	34	2	2	3	4	3	4	5	7	10	15	20
Fraction Action	Add 2ml RB	1	2	3	4	5 RB Change	6	7	8	9	10 RB Change	11	12

Table 4.2: The fractionation protocol with the additional step at the start. This method was followed during the molecular weight separation of the serum.

The cathode and anode reservoirs were filled with 6ml and 8ml of HEPS running buffer (RB) respectfully. The samples were added to a washed loading chamber, and 150µl of RB was added to the washed collecting chamber. The programme was started and after the 1st step, an additional 2ml of RB was added to the cathode reservoir, the loading chamber was washed twice with 200µl of RB and the programme continued. After each step, the resultant fraction was collected from the collection chamber, the chamber washed twice with 200µl of RB, re-loaded with 150µl of RB and the programme continued. Where indicated in Table 4.2, the RB was changed in both the anode and cathode reservoirs and the loading chamber also. After all the fractions were collected the cartridge was discarded.

4.2.1.7 1D SDS PAGE (1DE)

To evaluate the fractions a 1DE gel was performed on all fractions from the second duplicate fractionation, including the first three that were estimated to have low protein concentrations. Firstly 5µg of protein from fractions 4-12 and all of the protein within fractions 1-3 underwent a trichloroacetic acid (TCA), Na Deoxycholate (DOC) and acetone precipitation as follows. A 2% DOC solution was added to each sample to obtain a final concentration of 0.02% of the volume and left at RT for 15 minutes, before 100% TCA was added to obtain a final volume of 10% TCA before vortexing and a 1 hour RT incubation. The samples were centrifuged at 4ºC for 10 minutes, the supernatant removed and the pellet air dried. To each pellet 200µl of -20ºC acetone was added on ice, the samples incubated for 15 minutes, centrifuged at 15,000g at 4ºC for 10 minutes and the pellet air dried, after which the pellet was re-suspended in 10µl of Laemelli buffer. The samples were heated at 100ºC for 10 minutes before being pipetted alongside side both molecular weight marker (Novex See Blue Plus standard 1089578) and a whole serum sample of the fractionated sample containing 5µg of protein which underwent the same preparation as the fractions. The samples were loaded into different wells of a NuPAGE 4-12% Bis-Tris Midi gel (Novex Life Technologies WG1401BX10), the gels were loaded into a Introgen Novex Midi Gel system and ran in a Nu PAGE MED SDS running buffer (NP0002) for 1 hour at 200V. The gels were then fixed with EtOH 50%, H_3PO_4 3% before being stained with a colloidal Coomassie (MeOH 34%, $H_3PO_4$3% $(NH_4)2SO_4$ 17%).

4.2.1.8 Fraction analysis

The protein concentrations of fractions 1-12 (initial fractionation) and 4-12 (second fractionation from pools 1 and 2 in duplicate) was ascertained using RC DC protein assay kit (BioRad 500-0119) as previously described though no dilutions of the fraction samples took place. Fractions 1-3 of the second fractionation experiment were not quantified owing to the low concentration seen on the initial fractionation, and the fact that the protein needed to be preserved for both gel electrophoresis and mass spectrometry. Fractions 1-6 of both groups 1 and 2 were trypsin digested as previously described, with volumes adjusted for protein concentration and the addition of 30µl of ammonium bicarbonate to the final pellet. Each fraction underwent shotgun analysis as previously described and the resultant protein matches pooled to create a description of all proteins identified in fractions 1 – 6 from pool-1 and pool-2.

4.2.1.9 Shotgun analysis of immobilised p-aminophenyl phosphoyl choline gel elution

Early attempts at identifying CRP using an immobilised p-aminophenyl phosphoyl choline gel, which uses the affinity of CRP isolate it from serum resulted in an elution containing a number of proteins including a 25kDa protein. This elution underwent the same preparation as the fractions and underwent shotgun analysis with the aim of identifying the proteins in this sample.

4.2.2 Measurement of APPs and identification and measurement of other biomarkers of the acute phase response using quantitative proteomics

4.2.2.1 Identification and measurement of acute phase proteins using single ion monitoring SIM targeted MS/MS

4.2.2.1.1 Identifying acute phase proteins in initial shotgun analysis of pools

The shotgun analysis data, utilising the peaks from the MS/MS spectra was analysed. The peak area value derived from area under the curve (AUC) was used to give a relative quantification of each protein from each pool once the AUC values were normalised using intensity. Known APPs identified in the shotgun analysis of the HAP, AP and NAP pools (pools1-3) were targeted for quantification (with the exception of AGP, which was identified but not selected for measurement). The targeted APPs are detailed in Table 4.3. C-reactive protein was not selected as it was not identified in the initial shotgun analysis.

Acute phase protein	Abbreviation
Serum Amyloid A	SAA
Apolipoprotein AI	Apo-AI
Ovotransferrin	Ovt
Transthyretin	Ttn
Haemopexin	Hx
PIT54	PIT54

Table 4.3: Acute phase proteins targeted for quantification. These APPs, identified in the shotgun analysis, are known APPs and (with the exception of Apo-AI and Ttn) have been previously characterised in chickens. These were targeted for subsequent quantification.

4.2.2.1.2 Measuring acute phase proteins using data dependant single ion monitoring targeted MS/MS in three acute phase groups

Once the APP targets were selected, proteotypic peptides were assessed and quantotypic peptides identified. The samples from each pool were then assessed separately. Three HAP (pool-1) samples, and four AP and NAP (pools-2 and 3) samples were randomly selected. This shotgun analysis utilised the nano UPLC - Q-Exactive hybrid Quadrupole-Orbitrap mass spectrometre (Thermo Scientific) (data dependant single ion monitoring SIM/targeted MS/MS) in a targeted mode, targeting the peptides of each of the selected proteins, based on the results of the previous shotgun analysis.

The MS data was collated and following normalisation, analysed using Skyline v.3.5 (MacCoss Lab software). The MS/MS peaks were integrated and the AUC for each peptide (peptide intensity) was determined. The peptide intensities (the

sum of all the fragment ions of that peptide) were used to determine the intensities of each APP in each sample. For each APP, two, three or four peptide intensities were used and non-parametric Kruskal-Wallis tests were used to compare the peptide intensity for each selected peptide across the three sample groups and identify significant differences. Spearman's correlations were used to calculate any correlation in the area generated from each peptide of each APP. Statistical analysis was undertaken using GraphPad Prism v.5 and v.6.

4.2.2.1.3 Quantifying ovotransferrin and serum amyloid A by immunoassay

Using a radial immunodiffusion method described in Chapter 3, Ovt was measured in the HAP, AP and NAP samples. Serum amyloid A was measured using a commercial kit, acquired from Life Diagnostics (3400-7 Life Diagnostics, Stoke-on-Trent, UK) which utilises a chicken specific enzyme-linked immunoassay assay method (ELISA). This ELISA kit was used according to the manufacturer's instructions, with modifications to the initial serum concentrations, to reduce the volume of chicken sera used, and the serum was diluted 20 fold with the diluent, to obtain results that were measureable on the standard curve. By this stage of the work the third HAP sample was exhausted and as such no measurement is available for this sample. The results of the immunoassays were correlated with the peak area for each SAA and Ovt peptide generated from the targeted SIM/MS/MS results using a Spearman's correlation in GraphPad Prism v.6.

4.2.2.2 Identification and measurement of other biomarkers of the acute phase response using single ion monitoring targeted MS/MS

4.2.2.2.1 Identifying novel biomarkers of the acute phase response in the initial shotgun analysis of pools

Identification of other proteins in the serum that were differentially abundant between acute phase groups (pools) was undertaken to identify other possible biomarkers of the APR in chickens. To do this the peak AUC for all proteins identified in all three pools were normalised and the fold difference calculated between each pool for each protein and ranked to identify the most differentially abundant. From this shortlist proteins were further selected based

on the availability of suitable MS/MS data for targeted measurement generated from the shotgun analysis and the quantotypic nature of the constituent peptides of these proteins.

4.2.2.2.2 Measuring novel biomarkers of the acute phase response using data dependant single ion monitoring targeted MS/MS in three acute phase groups

Following the same protocol as detailed for APPs in 4.2.2.1.2, the novel proteins selected were quantified for the three highly acute phase and four acute phase and non-acute phase samples to determine whether these proteins were indeed differentially expressed between acute phase groups. The results were analysed as per Section 4.2.2.1.2.

4.2.3 Statistics

GraphPad Prism v.5 and v.6 were used for statistical analysis. All results were analysed using non-parametric tests: Kruskal-Wallis test with Dunn post-test analysis and Spearman's correlation, following data evaluation using D'Agostino-Pearson omnibus normality test. Non-parametric analysis was selected based on the results together with the small number of replicates in each group.

4.3 Results

4.3.1 Enrichment and shotgun analysis of acute phase serum

4.3.1.1 Initial attempts at identifying C-reactive protein in chicken serum

With the aim of purifying and identifying CRP in chicken serum, Western blotting using two α-CRP antibodies to identify any binding to chicken acute phase and non-acute phase serum was undertaken and the Western blot is shown in Figure 4.1.

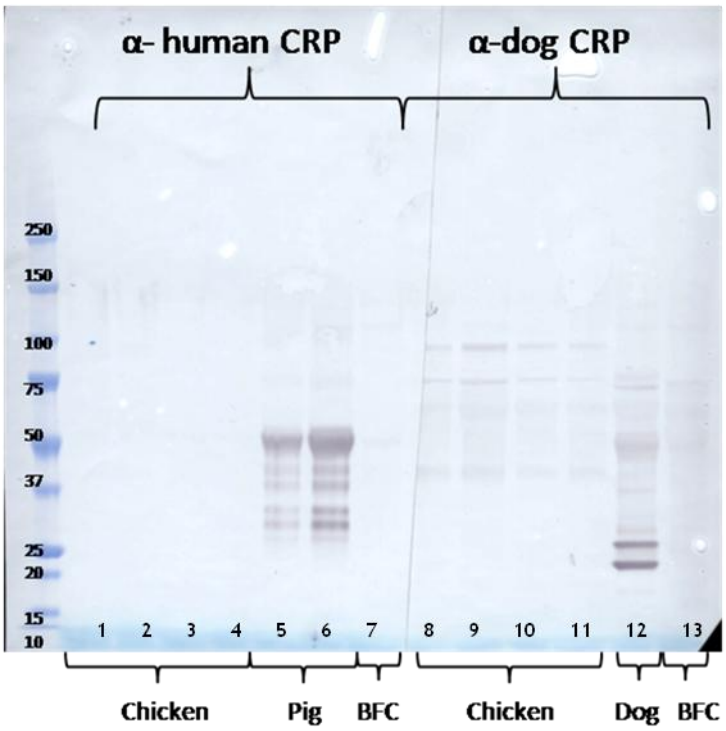


Figure 4.1: Western blot to detect cross reactivity of rabbit anti-human CRP and sheep anti-dog CRP antibodies to chicken serum (lanes 1-4 and 8-11). Both acute phase chicken serum (lanes 1-2 and 8-9) and non-acute phase serum (lanes 3-4 and 10-11) were used. Pig serum (lanes 5-6) and dog serum (lane 12) were also included on the western blots for the anti-human CRP and sheep anti-dog CRP antibodies respectfully. Separation of proteins was achieved using a 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124).

The western blots revealed no reactivity between the rabbit anti-human CRP antibody and the proteins in chicken serum. The human antibody shows cross reactivity with the pig serum and unspecific binding as well. The sheep anti-dog

CRP antibody has shown unspecific binding, most likely immunoglobulin within the chicken serum but no binding was seen at 25kDa the molecular weight of CRP.

Acute phase serum was injected onto an immobilized p-aminophenyl phosphoryl choline gel column (Thermo Scientific 20307) and following concentration of the eluted protein ran on a 1DE gel, alongside purified dog CRP. In Figure 4.2 a number of bands, typical in appearance of unspecific binding can be seen in the elution in lanes (1-6), including a single band at 25kDa. In lanes 7-9 the purified dog CRP can be seen as a double bands as two of the five CRP subunits are glycosylated and appear separately from the other subunits (Ceron *et al.*, 2005).

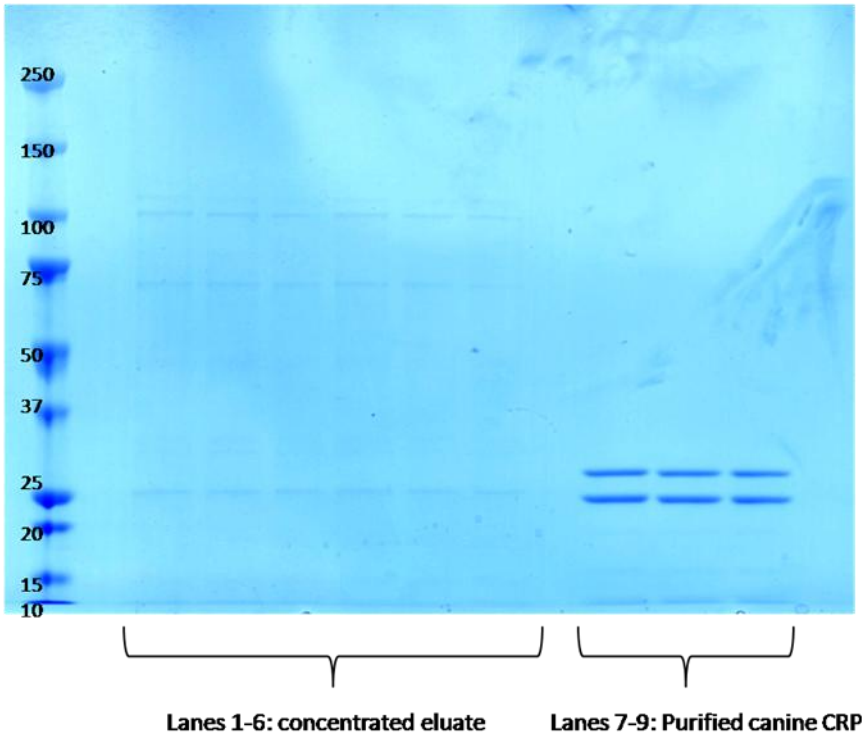


Figure 4.2: 1DE of concentrated eluate from FPLC and concentrated canine CRP. Separation was achieved using a 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124). Protein is stained with Coomassie blue G dye (Sigma Aldrich).

This band of interest from the elution together with 6 other bands were excised from the gel and identified using mass spectrometry. The excised bands and the identified proteins are detailed in Figure 4.3 and Table 4.4 respectfully.

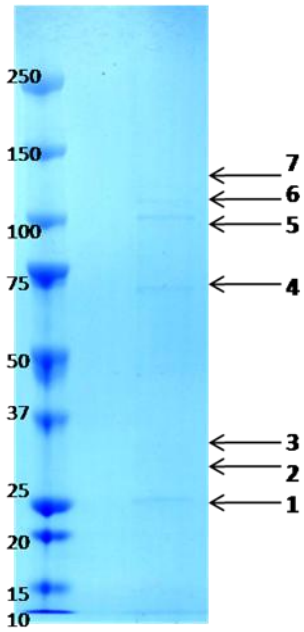


Figure 4.3: Bands present and excised from 1DE shown in Figure 4.2.

ID	Protein ID	Accession	Mr (da)	PI	Peptides matched	Sequences	Coverage %	Mascot Score
1	Apolipoprotein AI (*G. gallus*)	gi|211159	30673	5.58	15(4)	14(4)	51	145
	PREDICTED C-reactive protein (*Canis lupis familiaris*)	gi|345797697	25367	-	2(0)	2(0)	-	42
3	Predicted alpha tectorin like isoform 1 (*G.gallus*)	gi|363745877	29865	5.19	4(0)	4(0)	15	42
4	Serum albumin precursor (*G.gallus*	gi|45383974	69872	-	13(0)	11(0)	-	53
	Immunoglobulin (*G. gallus)*	gi|118088308	53607	6.84	4(1)	3(1)	6	50
	Plasminogen (*G. gallus*)	gi|118088308	49206	5.26	3(1)	3(1)	3	49

Table 4.4: Proteins identified in bands 1, 3 and 4 from Figure 4.3. Bands 2, 5, 6 and 7 were contaminants (human keratin). Proteins were identified by Mascot *Gallus gallus* peptide database (Blanks exist where Mascot updates have deleted original data). Bands were excised from the gel trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates. Both NCBI and where Uniprot identifications are listed (immunoglobulins are not listed in Uniprot). The protein score is the sum of the highest ions score for each distinct sequence. The first number under matches is the count of MS/MS spectra that have been matched to peptides from this protein. The matches shown in the parenthesis indicating unique matches (scores above the significance threshold). The sequences column is the count of matches to distinct peptide sequences, the number in

parenthesis is the count of sequences with significant matches (Matrixscience.com). The coverage percentage is proportion of the protein's entire sequence identified.

Band 1, the most intense band was identified as apolipoprotein AI (Apo-AI), the major protein fraction of high-density lipoproteins (HDL). There were no matches for CRP or other pentraxans to *Gallus gallus*. When the search was widened to include all boney vertebrates there was a predicted match to canine CRP identification for two peptides. Given the low score and the presence of purified and highly concentrated dog CRP on the gel, this is highly likely to be contamination. Other bands excised were identified as human keratin a gel contaminant.

4.3.1.2 Protein fractionation

Two fractionations were undertaken using the Protein Discovery GelFree® 8100 Fractional system (Expedeon, Cambridge, UK) to enrich the lower molecular weight proteins within HAP (pool-1/HAP) and AP (pool-2/AP) pooled serum samples to identify low abundant SAA and CRP by shotgun analysis. Initially a single sample from pool-1/HAP was fractionated and the protein concentrations of each fraction determined. Table 4.5 contains the estimated molecular weight of each fractionation based on the manufactures instructions. Figure 4.4 is the 1DE gel containing the 12 fractions produced from pool – 1/HAP. Overlaid in graphical form are the protein concentrations of each fraction. Owing to the low concentration of protein at the lower molecular weights and because the protein concentrations did not appear to correlate with the band intensity this experiment was repeated, this time using both pools 1 and 2 in duplicate to assess the reproducibility of the method.

Fraction	Estimated molecular weight of proteins (kDa)
1	6 – 14
2	8-15
3	15-19
4	18-26
5	23-31
6	28-34
7	34-38
8	37-50
9	40-53
10	50-55
11	Around 60
12	Around 66

Table 4.5: Estimated molecular weight of each fraction according to Protein Discovery GelFree® 8100 Fractional system instructions (Expedeon, Cambridge, UK).

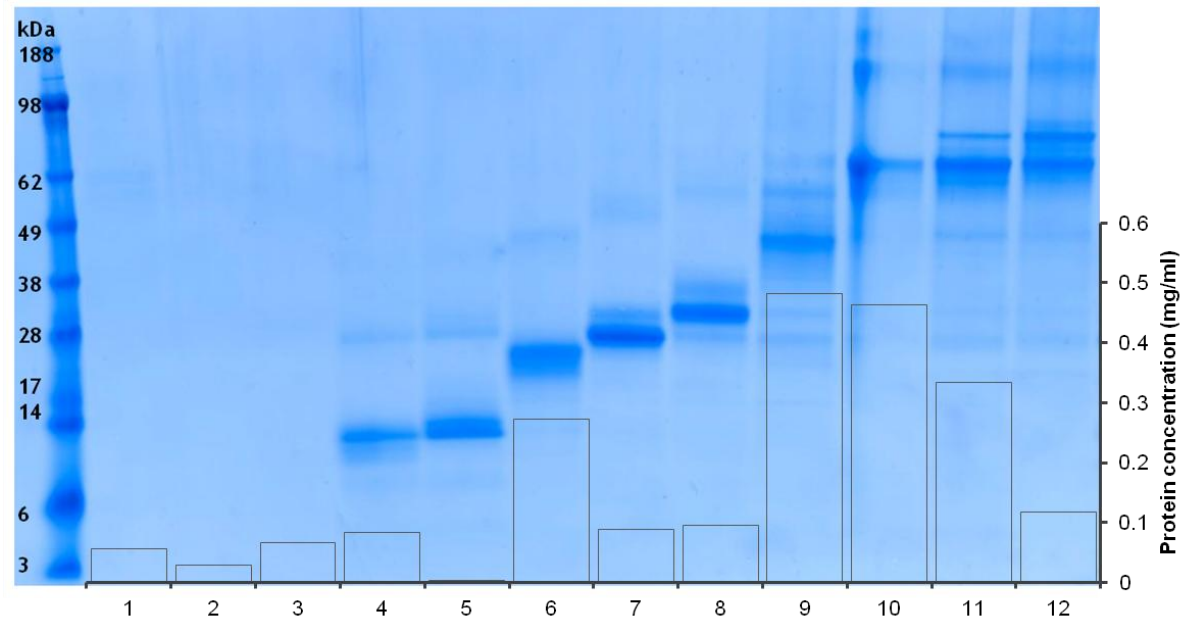


Figure 4.4: 1DE gel containing the 12 fractions collected from the fractionation of pool-1 serum. The superimposed graph shows protein concentration of each fraction. Protein separation was achieved using NuPAGE 4-12% Bis-Tris Midi gel (Novex Life Technologies WG1401BX10) and protein concentrations determined using RC DC protein assay kit (BioRad 500-01190).

No protein quantification was undertaken for the first three fractions of the second fractionation experiment as it was previously determined that the protein concentrations of these fractions was very low and would need to be preserved for the following shotgun analysis. Based on the estimated molecular weight expected from the initial fractions, the 1st fraction of the second fractionation experiment was split into two and recovered separately and the final fraction was omitted. Figure 4.5 shows the protein concentrations of each of the 8 fractions from pools 1 and 2 in duplicate. All four sample fractions show a similar trend, albeit the first duplicate from pool-2 appears to show the same trend but one fraction prematurely to the other fractions, indicating that the method of separating proteins according to their molecular weight was effective.

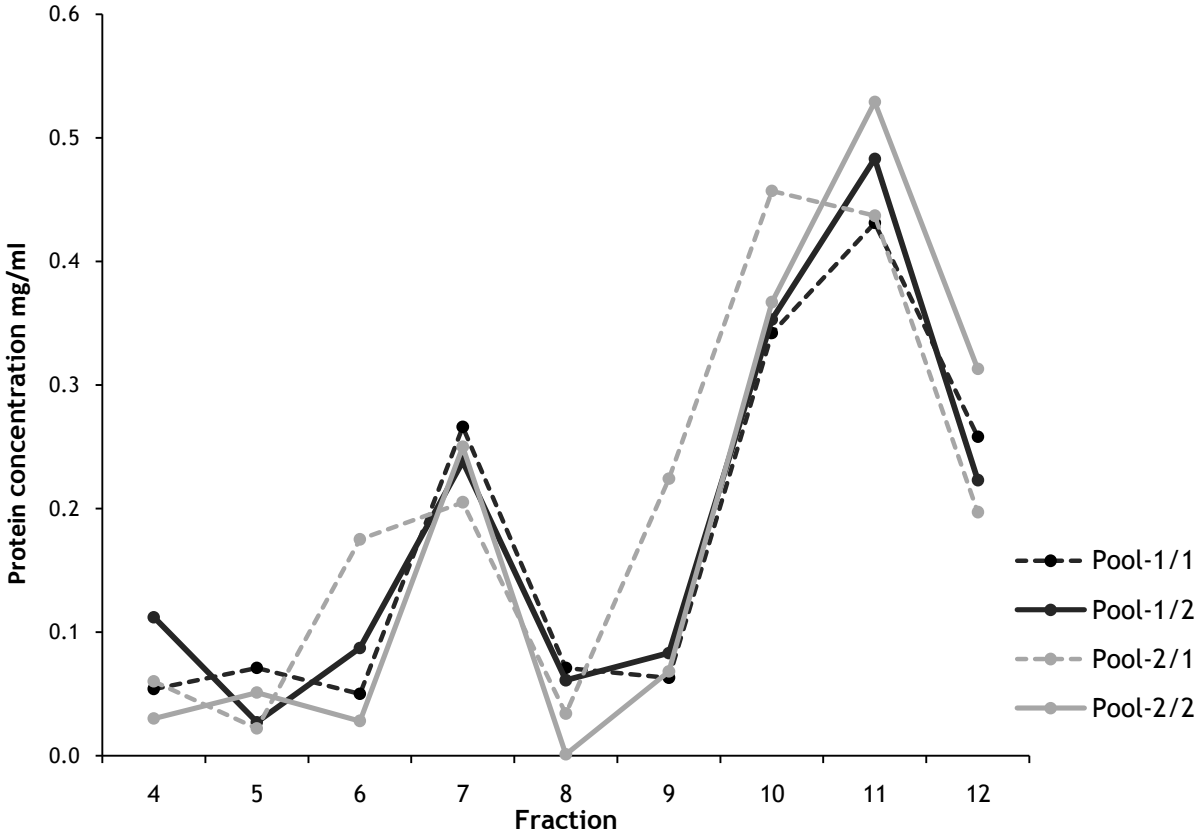


Figure 4.5: Protein concentrations of fractions 4-12 of pool-1 and pool-2 duplicate fractionations. Protein concentrations, determined using RC DC protein assay kit (BioRad 500-01190), were plotted (y-axis) against fraction number (x-axis) for each of the two samples, ran in duplicate.

4.3.1.3 Shotgun analysis: Identifying Serum amyloid A and C-reactive protein

4.3.1.3.1 Serum

Shotgun analysis was performed on three acute phase status serum pools: HAP (pool-1), AP (pool-2) and NAP (pool-3). The results were analysed using both Mascot (Matrix Science) and SEQUEST (Thermo Finnegan) software. A total of 671, 591 and 592 proteins from pools 1, 2 and 3 were identified. Figure 4.6, a Venn diagram comparing proteins identified in each pool, reveals that each pool contains a significant number of proteins not shared with the other two pools and 242 proteins were common between all three pools. Serum amyloid A was identified in pool-1, the HAP serum from viraemic birds and pool-2 from AP birds. Serum amyloid A was not detected in pool 3, the NAP pool. Table 4.6 details the SAA peptides isolated from the pool-1 and pool-2 serum. The protein results were filtered and a minimum of two peptides required for a significant protein identification. When filter criteria was relaxed a single CRP peptide was identified in pool-2. This however is not sufficient to make a positive identification of CRP in the serum.

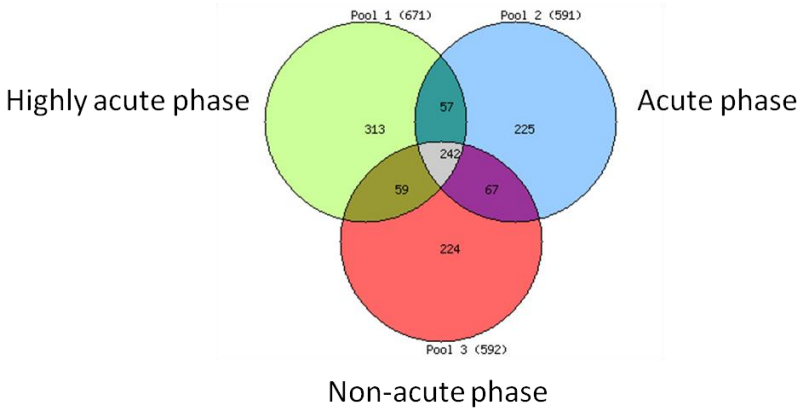


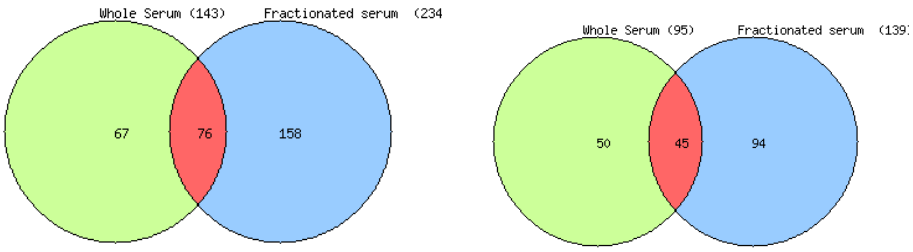
Figure 4.6: Venn diagram of proteins identified by shotgun analysis of pools 1-3 (HAP, AP and NAP). Of the total number of proteins identified 242 were the same, with the HAP (pool-1) having the largest number of unique proteins.

Pool	Peptide sequence	Accession	Protein	Missed	Database
Pool-1	EANYIGADKYFHAR	F1NW65	SAA	1	SEQUEST
	LDQEANEWGR			0	
	GPGGAWAAK			0	
	FRPAGLPNK			0	
	FRPAGLPNKY			1	
Pool-2	EANYIGADKYFHAR	F1NW65	SAA	1	SEQUEST
	LDQEANEWGR			0	
Pool-3	SYTDLTRPHSLFSYATK	Q2EJU6	CRP	0	SEQUEST

Table 4.6: Peptides identified from SAA and CRP from whole serum shotgun analysis.

4.3.1.3.2 Fractions

The shotgun analysis results for the first six fractions from pool-1 and pool-2 were consolidated for each pool. To compare the numbers of proteins identified in the whole serum and fractionated serum to assess whether the fractionation process did indeed enrich lower molecular weight proteins, proteins over 38kDa from both shotgun result sets were excluded and a comparison made. Figure 4.7 contains the Venn diagrams for each pool.



Pool-1 Pool-2

Figure 4.7: Venn diagrams comparing the number of proteins that are less that 38kDa identified in whole serum and fractionated serum, in pool-1 (left) and pool-2 (right).

The fractionated serum, enriched to exclude higher molecular weight proteins, including highly abundant albumin and IgG, and increase the concentration of the lower molecular weight proteins within a sample, resulted in a further 158

and 94 proteins being identified from the pool-1 and pool-2 samples respectfully, indicating that the enrichment technique was successful. It is clear that the fractionation was especially successful on pool-1, the HAP sample, with the identification of 233 proteins in this pool compared to the 139 identified from pool-2. It was also evident that the fractionation process resulted in the loss of lower molecular weight proteins, with 67 and 50 proteins identified only in the whole serum of pools 1 and 2 respectfully. Analysis of the proteins present in the fractionated samples from pools 1 and 2 resulted in the identification of a larger number of SAA peptides being identified in the pool-1 and 2 samples.

Pool	Peptide sequence	Missed cleavages	Accession	Protein	Database
Pool -1	GAEDTRLDQEANEWGR	1	F1NW65	SAA	SEQUEST
	LDQEANEWGR*	0			
	EANYIGADKYFHAR*	1			
	EANYIGADK	0			
	VSGRGAEDTRLDQEANEWGR	2			
	RGPGGAWAAK	1			
	FRPAGLPNK*	0			
	GPGGAWAAK*	0			
	LDQEANEWGRR	1			
	FRPAGLPNKY*	1			
	DAAGGARDMWRAYR	2			
Pool - 2	GAEDTRLDQEANEWGR^{+}	1	F1NW65	SAA	SEQUEST
	LDQEANEWGR**	0			
	EANYIGADKYFHAR**	1			
	RGPGGAWAAK^{+}	1			
	YFHARGNYDAARR	2			
	DAAGGARDMWRAYR	2			
	GNYDAARRGPGGAWAAK	2			
	FRPAGLPNKY**	1			

Table 4.7: SAA peptides identified by shotgun analysis of fractions 1-6 of Pools 1 and 2.
*Also identified in the serum shotgun analysis of pool-1. **Also identified in pool-1 serum.
$^{+}$Also seen in pool-1 fractionated results.

No CRP peptides were identified in the fractionated shotgun results from pool-1 or pool-2, even when the search criteria was relaxed. Table 4.7 lists the identified peptides from the fractionation shotgun analysis.

4.3.1.4 Shotgun analysis of immobilised p-aminophenyl phosphoyl choline gel elution

With the aim of identifying CRP in chicken serum, the elution from the immobilised p-aminophenyl phosphoyl choline gel underwent shotgun analysis alongside the fractions. No CRP or pentraxin peptides were identified.

4.3.2 Measurement of APPs and identification and measurement of other biomarkers of the acute phase response using quantitative proteomics

4.3.2.1 Identification and measurement of acute phase proteins using single ion monitoring SIM targeted MS/MS

4.3.2.1.1 Identifying acute phase proteins in initial shotgun analysis of pools

The data from the shotgun analysis on the whole serum from pool-1 and pool-2 was used to calculate relative quantification of the SAA protein. Using the peak area and normalising using intensity, the relative concentrations between pools 1, 2 and 3 were determined. Pool-3 samples from the NAP birds contained no peptides from SAA. Pool-1 from the HAP viraemic birds was shown to be 5.8 fold higher than in the pool-2 samples from AP birds making this a target for further investigation and quantification.

Other known APPs were identified from the shotgun analysis results and the fold difference between the quantity of these proteins between the HAP, AP and NAP pools determined. Table 4.8 and Figure 4.8 detail the fold differences between the pools of each of these APPs.

Acute phase Protein	HAP/ AP	AP/ NAP	HAP/ NAP
Haemopexin	-1.063	1.147	1.079
Transthyretin	-3.070	1.106	-2.775
α- 1-Acid glycoprotein	1.318	2.143	2.824
PIT 54	-1.097	1.270	1.158
Ovotransferrin	2.158	1.025	2.211
Apolipoprotein A-I	-2.499	1.326	-1.885
SAA	5.891	*	*

Table 4.8: Acute phase proteins identified and the fold difference in peak area between these APPs in the three acute phase pools. * SAA was not detected in AP and NAP pools therefore no fold difference calculation could be made.

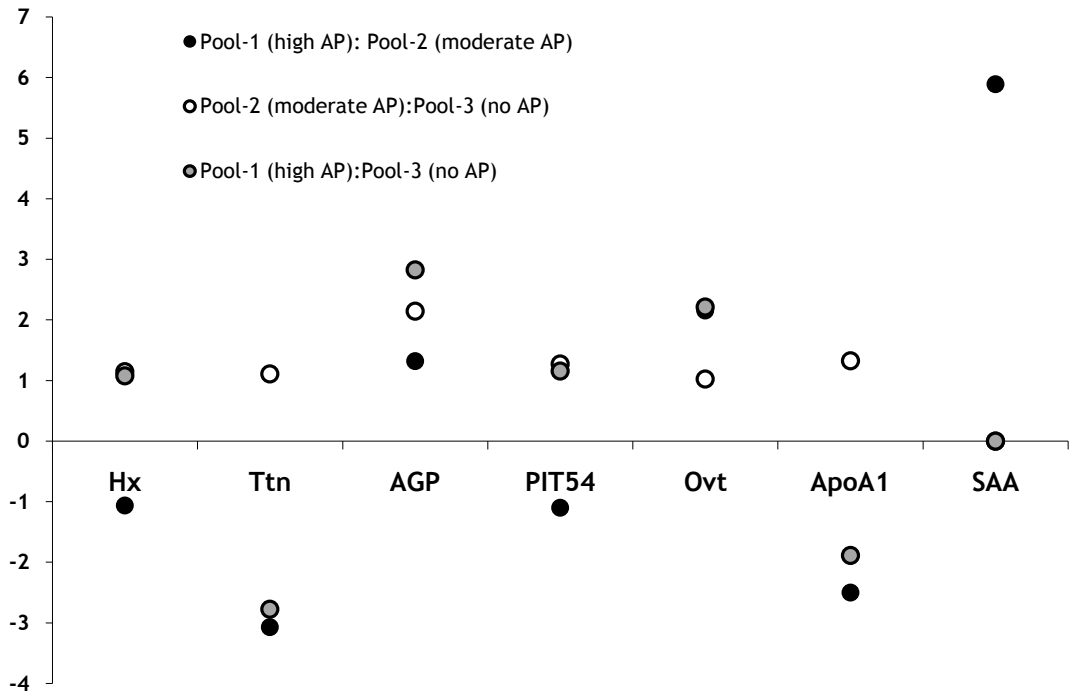


Figure 4.8: APP fold differences between three acute phase pools. Each point indicates the fold difference between the pool listed left in the legend relative to the pool listed right in the legend.

4.3.2.1.2 Measuring acute phase proteins using data dependant single ion monitoring targeted MS/MS in three acute phase groups

The APPs SAA, Ovt, Apo-AI, Hx, Ttn and PIT54 were selected to be measured by targeted SIM MS/MS to determine whether these APPs could be measured using this method and to measure the relative differences in abundance between each of the acute phase groups. A minimum of three peptides are required for significant identification and measurement of an APP, for Hx only two suitable peptides were identified but this APP was still included for analysis. Table 4.9 details the statistical analysis of the peak areas for all measured peptides for each APP. A Kruskal-Wallis test was applied to each of the APP peptides with Dunn's post test analysis. Figure 4.9 gives a graphical representation of the results of each APP. To assess the success of the SIM targeted MS/MS method for measuring these chicken APPs, a Spearman correlation between each of the peptide results was made for each APP.

Protein	**Statistical test**	**Peptides**			
		Peptide 1	Peptide 2	Peptide 3	Peptide 4
Serum Amyloid A F1NW65		R.EANYIGADK.Y [47, 55]	R.EANYIGADKYFHAR.G [47, 60]	R.LDQEANEWGR.R [100, 109]	
Kruskal-Wallis test (p-value)		0.0158	0.0465	0.0078	
Dunn post-test analysis	High AP vs AP	Ns	ns	*	
	High AP vs Non-AP	*	*	*	
	AP vs Non-AP	Ns	ns	ns	
Spearman Correlation		*p-value*	r^2*-value*		
*Peptide 1 and 2		0.0225	0.6707		
**Peptide 1 and 3		0.0010	0.9106		
*Peptide 2 and 3		0.0121	0.776		
Ovotransferrin P02789		R.GAIEWEGIESGSVEQAVAK.F [154, 172]	K.VEDIWSFLSK.A [278, 287]	K.YFGYTGALR.C [539, 547]	K.FYTVISSLK.T [679, 687]
Kruskal-Wallis test (p-value)		0.0116	0.1551	0.0163	0.0282
Dunn post-test analysis	High AP vs AP	Ns	ns	Ns	Ns
	High AP vs Non-AP	**	ns	*	*
	AP vs Non-AP	ns	ns	Ns	Ns
Spearman Correlation		*p-value*	r^2*-value*		
****Peptide 1 and 2		<0.0001	0.9545		
****Peptide 1 and 3		<0.0001	0.9229		
**Peptide 1 and 4		0.0253	0.6788		
***Peptide 2 and 3		0.0004	0.8793		
*Peptide 2 and 4		0.0371	0.6424		
**Peptide 3 and 4		0.0010	0.8454		
Apolipoprotein AIP08250		K.DAIAQFESSAVGK.Q [50, 62]	K.LADNLDTLSAAAAK.L [68, 81]	K.WTEELEQYR.Q [130, 138]	
Kruskal-Wallis test		0.0406	0.0282	0.0346	

Dunn post-test analysis	High AP vs AP	*	*	*	
	High AP vs Non-AP	ns	ns	Ns	
	AP vs Non-AP	ns	ns	Ns	
Spearman Correlation		*p-value*	*r-value*		
	****Peptide 1 and 2	<0.0001	0.9636		
	****Peptide 1 and 3	<0.0001	0.9636		
	****Peptide 2 and 3	<0.0001	0.9727		
Haemopexin H9L385		R.QDHPEEHQSLYLFQDEK.V [68, 84]	K.VFSFDLELR.V [136, 144]		
Kruskal-Wallis test (p-value)		0.1137	1.0000		
Dunn post-test analysis	High AP vs AP	ns	ns		
	High AP vs Non-AP	ns	ns		
	AP vs Non-AP	ns	ns		
Spearman Correlation		*p-value*	*r^2-value*		
	Peptide 1 and 2	ns	0.5182		
Transthyretin P27731		K.AADGTWQDFATGK.T [58, 70]	R.VEFDTSSYWK.G [93, 102]	R.HYTIAALLSPFSYSTTAVVSDPQE.- [126, 149]	
Kruskal-Wallis test (p-value)		0.0400	0.0697	0.1400	
Dunn post-test analysis	High AP vs AP	*	Ns	Ns	
	High AP vs Non-AP	*	Ns	Ns	
	AP vs Non-AP	*	Ns	Ns	
Spearman Correlation		*p-value*	*r^2-value*		
	****Peptide 1 and 2	<0.0001	0.9455		
	*Peptide 1 and 3	0.0128	0.7182		
	**Peptide 2 and 3	0.0062	0.7636		
PIT54 H9KZK6		R.VEVLHNDVWGTVCDEGWDLR.E [79, 98]	K.QLGCGDAVLAPIAAK.F [216, 230]	R.VEVLHNNVWGTVCDDNWDLR.E [299, 318]	R.GPDVIWLDDVNCEGTEESIFDCK.A [454, 476]
Kruskal-Wallis test (p-value)		0.4169	0.2335	0.4060	0.8401
Dunn post-test analysis	High AP vs AP	ns	Ns	ns	ns
	High AP vs Non-AP	ns	Ns	ns	ns
	AP vs Non-AP	ns	Ns	ns	ns
Spearman Correlation		*p-value*	*r^2-value*		
	*Peptide 1 and 2	0.0289	0.6545		
	****Peptide 1 and 3	<0.0001	0.9091		
	*Peptide 1 and 4	0.0146	0.7091		
	**Peptide 2 and 3	0.0020	0.8091		
	**Peptide 2 and 4	0.0085	0.7455		
	*Peptide 3 and 4	0.0146	0.7091		

Table 4.9: Statistical results of SIM/targeted MS2 of acute phase proteins in three acute phase groups: HAP (n=3), AP (n=4) and NAP (n=4). (ns = no significance, *<0.05, **<0.01, ***<0.001, ****<0.0001). For each protein the quantitypic peptides are listed to the right in columns, underwhich the statistical results of the Kruskal-Wallis test are listed (p-value). The results of the Dunn post-test analysis, conducted to identify, where present, between which of the acute phase groups the significant difference lies are detailed in the rows underneath. Underneath is the result of the Spearman correlation, undertaken between the peptides for each protein across all the samples to determine whether the peptide peak areas (intensities) for each peptides, tryptically digested from the target protein correlate with each other. Asterisks to the the left of the peptide correlation indications level of significance, with p-value and r^2-value listed alongside.

All three of SAA's peptides were found to be significantly different between the three acute phase groups, and all three peptides correlated significantly with each other. Of the four peptides measured for Ovt, peptide 2 had no significant correlation with any other peptide and this peptide was the only peptide found not to be significantly different between the three acute phase groups. Apolipoprotein A-1 in this study behaves as a negative APP, with all three peptides showing significantly different peak areas, with the HAP Apo-AI peptides having significantly lower peak area than AP and NAP groups. These results suggest Apo-AI is a negative APP in chickens. Only two peptides were quantified for Hx and both revealed no changes in peak area between the acute phase groups. The HAP group had lower peak areas for all three Ttn peptides, with peptide one significantly lower than the other two groups. PIT54 was the only APP measured that shown no significant differences of peak area between any of the acute phase groups for any of the four peptides measured. Conversely this APP showed the greatest correlations between measured peptides indicating that while no significant differences between groups were found, the peptides measured were consistent in their peak area.

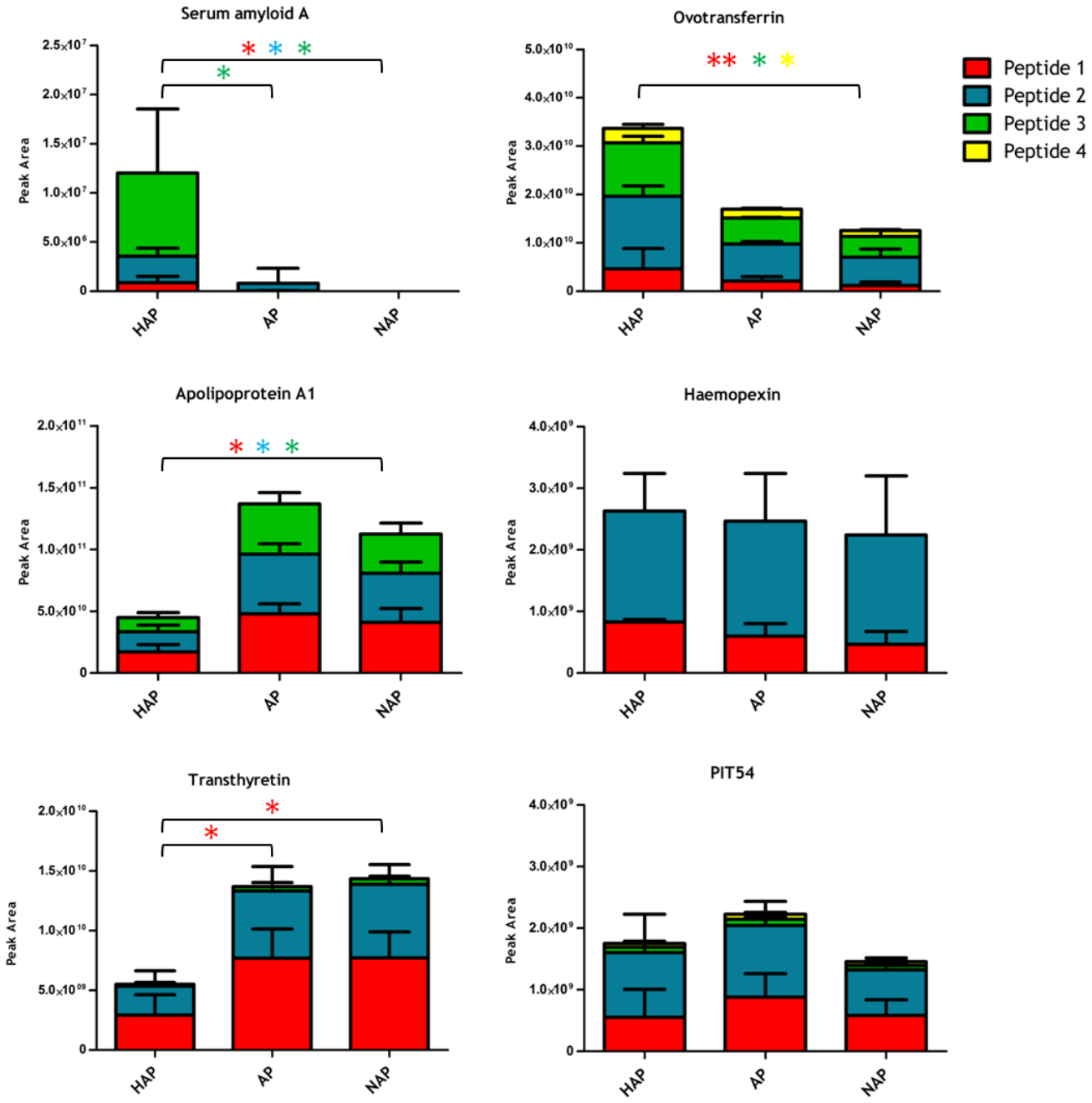


Figure 4.9: Peak area (± SD of the mean) of each APP, divided within bar by peptide in three different acute phase status groups: highly acute phase (HAP) (n=3), acute phase (AP) (n=4) and non-acute phase (NAP) (n=4). Statistical analysis was undertaken using Kruskal-Wallis with Dunn post-test analysis (GRaphPad Prism v5). Significance between groups (as detailed in Table 4.9) is indicated by asterisk *<0.05, **<0.01, ***<0.001, ****<0.0001, the colour of the asterisks corresponds to same coloured peptide as indicated in legend.

4.3.2.1.3 Quantifying serum amyloid A and ovotransferrin by immunoassay

Using the method described in Chapter 3 and a commercially available ELISA kit (3400-7 Life Diagnostics, Stoke-on-Trent, UK), Ovt and SAA was measured in the HAP, AP and NAP samples (with the exception of the third HAP sample, which had become exhausted). Non-parametric Kruskal-Wallis test was performed between each group and Spearman's correlations between the assay results and each of the peptides of the two proteins was performed and the results detailed in Table 4.10 and Figure 4.10.

Serum Amyloid A		**ELISA**	
Kruskal-Wallis (p-value)		0.0019	
Dunn post test paired analysis	HAP – AP HAP – NAP AP - NAP	Ns * Ns	
Spearman's correlation		*p-value*	*r-value*
	**Peptide 1	0.0028	0.8128
	*Peptide 2	0.0500	0.6487
	*Peptide 3	0.0222	0.7006
Ovotransferrin		**RID**	
Kruskal-Wallis (p-value)		Ns	
Spearman's correlation		*p-value*	*r-value*
	Peptide 1	0.0816	0.5836
	Peptide 2	0.1150	0.5350
	Peptide 3	0.0138	0.7599
	Peptide 4	0.1144	0.5335

Table 4.10: Statistical results of Ovt and SAA assays, together with correlations of each peptide to the assay results. For both SAA and Ovt the statistical results of the Kruskal-Wallis test is listed (p-value) together with the result of the Dunn post-test analysis, conducted to identify, for SAA, between which of the acute phase groups the significant difference lay. Underneath is the result of the Spearman correlation, undertaken between each peptide of SAA and Ovt and the ELISA and RID result respectfully. Asterisks to the the left of the peptide correlation indications level of significance, with p-value and r^2-value listed alongside.

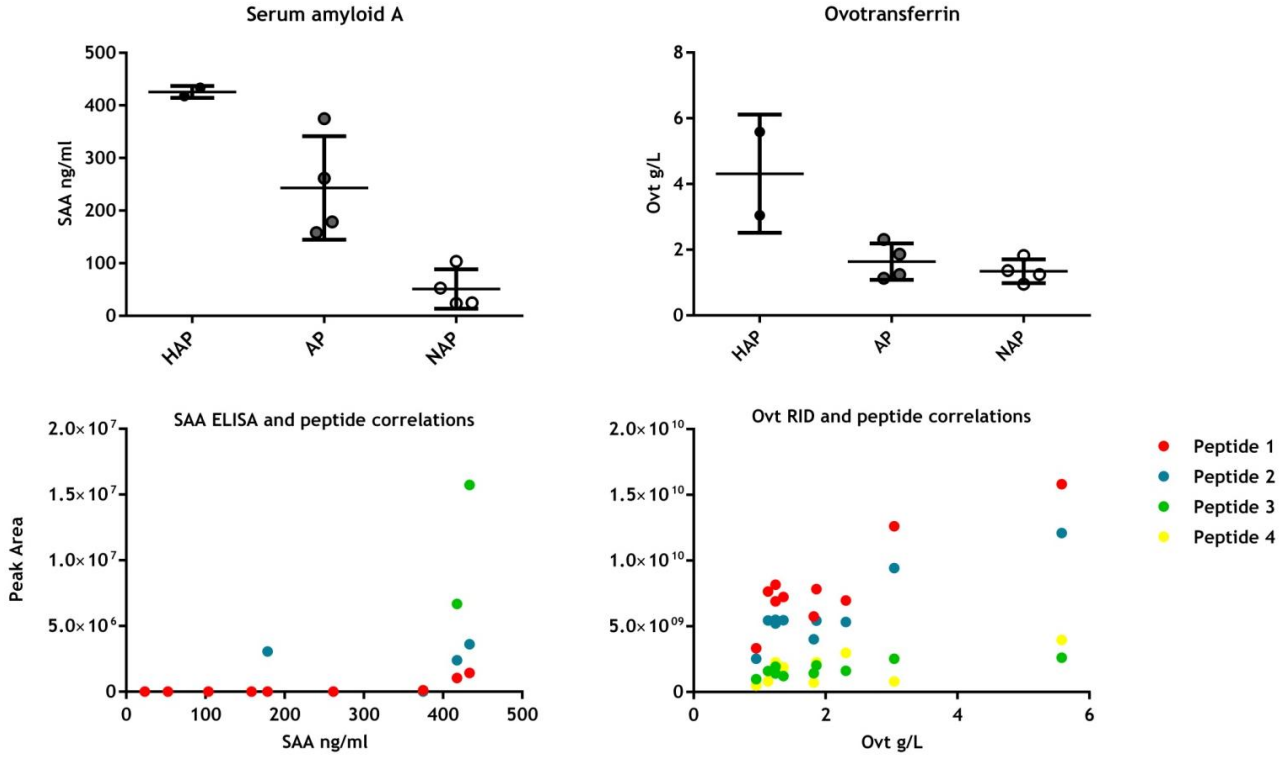


Figure 4.10: Scatterplots of SAA and Ovt concentrations in HAP (n=2), AP (n=4) and NAP (n=4), determined by ELISA and RID respectfully (first row). X-Y scatterplots of SAA and Ovt immunoassay result (x-axis) and the peak area of each peptide (y-axis) as determined by targeted SIM MS/MS (second row).

4.3.2.2 Identification and measurement of novel biomarkers of the acute phase response using single ion monitoring targeted MS/MS

4.3.2.2.1 Identifying novel biomarkers of the acute phase response in the initial shotgun analysis of pools

The results of the initial shotgun analysis of the three acute phase pools were used to identify other proteins that were differentially abundant between acute phase groups. A total of 1187 proteins were identified across the three acute phase pools of which 242 were identified in all three pools. The fold differences between the shared 242 proteins were ranked according to fold change. Figure 4.11 plots the fold differences between each of the 242 proteins between pools showing that smaller numbers of proteins are associated with larger fold differences between acute phase pools and the majority of shared proteins are

present in the three pools in similar quantities. Appendix 4.3 lists the proteins that show a greater than four-fold difference between each of the pools. A moderate APP shows a greater than four-fold difference during an APR and as such this was used as a cut off point to indicate that a protein could be differentially present. As this analysis excludes proteins that are absent from one of the three pools (such as SAA, which was absent from pool-3 the non-acute phase group), a further list was generated that included proteins present in the acute phase groups only (pools 1 and 2) but absent in the non-acute phase group (pool-3). This enabled the identification of proteins that are absent in a healthy non-acute phase state and as such potentially good biomarkers for the APR. Appendix 4.3 lists proteins that show greater than four-fold difference between these acute phase groups that are absent from pool-3. From this shortlist a selection was made based on availability of suitable MS/MS data for targeted measurement generated from the earlier shotgun analysis and the quantotypic nature of the constituent peptides of these proteins. Using this selection criteria 11 proteins were selected for targeted quantification, to determine whether these results were reflective of all samples within the pools and as such whether these were, as differentially expressed proteins, potential biomarkers of the APR. Two further proteins: myeloid protein (P08940) and an uncharacterised protein with no close homologies (FIP580), with smaller fold differences between groups were also selected given the availability of good MS/MS data. Table 4.11 lists the selected proteins.

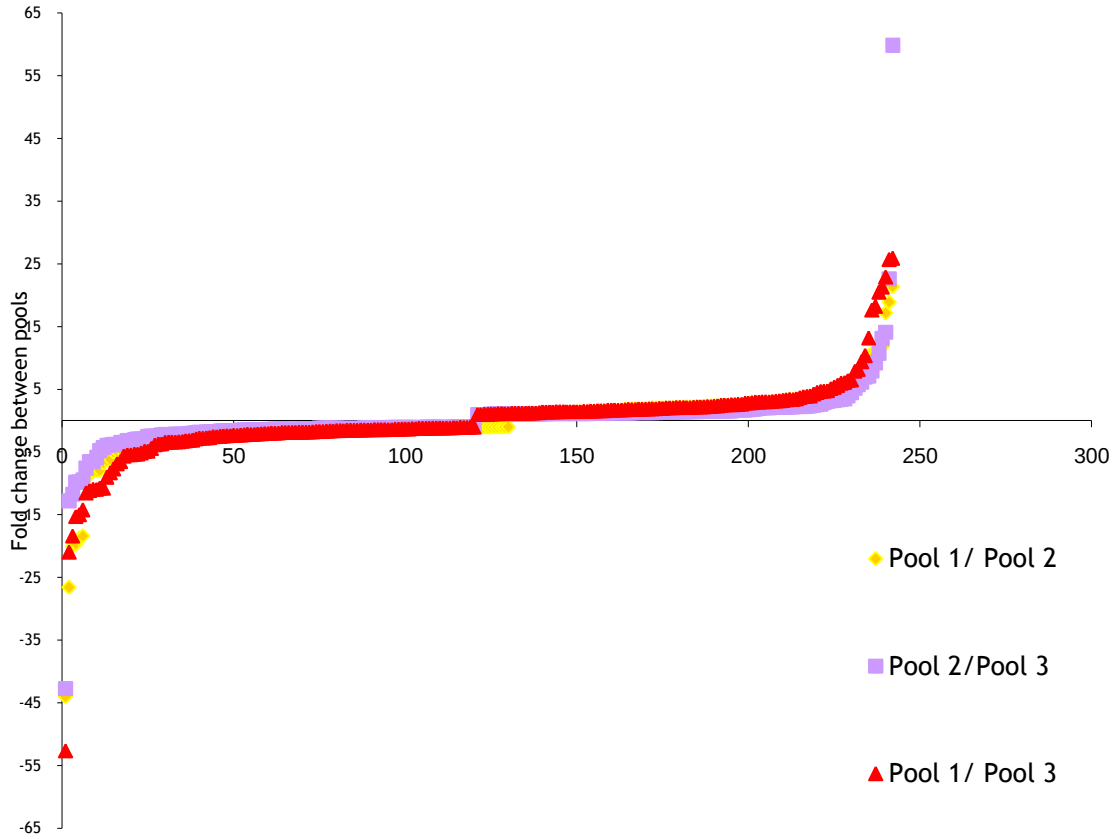


Figure 4.11: The fold difference of each protein identified from pools 1-3. The data from the shotgun analysis conducted on the three acute phase pools was normalised using intensity, and the fold difference for proteins identified in all three pools (n=242) was calculated. This was plotted (x-axis) between each of the groups.

Protein	Accession
Beta-2-Microglobulin	P21611
Coronin	Q5ZI60
Cathepsin D	Q05744
Matrilin-3	O42401
Myeloid Protein	P08940
Tenascin	P10039
Putative ISG12(2) protein	Q6IEC5
Hsp90 co-chaperone Cdc37	O57476
*Mannan-binding lectin associated serine protease 2	F1NAP7
*Alpha-2-HS-glycoprotein/fetuin-A	E1BZE1
*Major facilitator superfamily domain-containing protein 10	E1C932
*Complement C1r subcomponent	F1NAB7
No close homologies	F1P580

Table 4.11: Proteins selected for further quantification. *indicates an uncharacterised protein, the name of which is inferred from close homology.

4.3.2.2.2 Measuring novel biomarkers of the acute phase response using data dependant single ion monitoring targeted MS/MS in three acute phase groups

To investigate whether these differences are significant between individual samples the samples constituting the three pools were measured individually with all three HAP samples and four AP and four NAP samples selected randomly from the six samples included in pools 2 and 3. These underwent the same preparation and were measured in the same way as described for the APPs in Section 4.3.2. Table 4.12 details the results of the statistical analysis of the peak areas of the measured peptides of each of the selected proteins a Spearman correlation between each of the peptide results for each protein was also made. Figure 4.12 graphically details the results of each protein.

Protein	**Statistical test**	**Peptides**			
		Peptide 1	Peptide 2	Peptide 3	Peptide 4
Beta-2-Microglobulin P21611		R.LVHADFTPSSGSTYACK.V [84, 100]			
	Kruskal-Wallis test (p-value)	0.0406			
	Dunn post-test analysis: High AP vs AP	ns			
	Dunn post-test analysis: High AP vs Non-AP	*			
	Dunn post-test analysis: AP vs Non-AP	ns			
Coronin Q5ZI60		K.NDQCYDDIR.V [19, 27]	K.SDLFQDDLYPDTAGPEAALEAEEWFEGK.N [353, 380]	K.NADPLLISLK.H [381, 390]	
	Kruskal-Wallis test (p-value)	0.6276	0.7004	0.6181	
	Dunn post-test analysis: High AP vs AP	ns	Ns	ns	
	Dunn post-test analysis: High AP vs Non-AP	ns	Ns	ns	
	Dunn post-test analysis: AP vs Non-AP	ns	Ns	ns	
	Spearman Correlation	*p-value*	r^2*-value*		
	Peptide 1 and 2	ns	0.4273		
	**Peptide 2 and 3	0.0022	0.8364		
	Peptide 1 and 3	ns	0.4455		
Cathepsin D Q05744		K.LGFADLAEPTPEILK.N [56, 70]	R.DPTAQPGGELLLGGTDPK.Y [223, 240]	K.GGCEAIVDTGTSLITGPTK.E [275, 293]	
	Kruskal-Wallis test (p-value)	0.0308	0.0308	0.0463	
	Dunn post-test analysis: High AP vs AP	*	Ns	ns	
	Dunn post-test analysis: High AP vs Non-AP	ns	*	*	
	Dunn post-test analysis: AP vs Non-AP	ns	Ns	*	
	Spearman Correlation	*p-value*	r^2*-value*		
	Peptide 1 and 2	ns	-0.1273		
	**Peptide 2 and 3	0.0086	07636		
	Peptide 1 and 3	ns	-0.2182		
Matrilin-3 O42401		K.NRPLDLVFIIDSSR.S [49, 62]	K.VVIIVTDGRPQDQVENVAANAR.T [160, 181]	R.TAGIEIYAVGVGR.A [182, 194]	

Kruskal-Wallis test (p-value)		0.0487	0.0764	0.0498	
Dunn post-test analysis	High AP vs AP	*	ns	*	
	High AP vs Non-AP	ns	ns	*	
	AP vs Non-AP	ns	ns	ns	
Spearman Correlation		*p-value*	r^2*-value*		
	***Peptide 1 and 2	<0.0001	0.9729		
	**Peptide 2 and 3	0.0022	0.8364		
	**Peptide 1 and 3	0.0055	0.7909		

Myeloid Protein P08940		K.GVDVICTDGSIVYAPFSGQLSGPIR.F [68, 92]	K.GVDVICADGATVYAPFSGELSGPVK.F [218, 242]	K.FFHNGNAIDDGVQIR.G [243, 257]	R.VFPGIISHIHVENCDR.S [292, 307]
Kruskal-Wallis test (p-value)		0.1217	0.1217	0.0487	0.3423
Dunn post-test analysis	High AP vs AP	ns	ns	*	ns
	High AP vs Non-AP	ns	ns	*	ns
	AP vs Non-AP	ns	ns	ns	ns
Spearman Correlations		*p-value*	r^2*-value*		
	***Peptide 1 and 2	<0.0001	0.9818		
	***Peptide 1 and 3	<0.0001	0.9522		
	**Peptide 1 and 4	0.0018	0.8455		
	***Peptide 2 and 3	<0.0001	0.9704		
	***Peptide 2 and 4	0.004	0.9000		
	***Peptide 3 and 4	0.0007	0.8793		

Tenascin P10039		R.ESAPATINAGTDLDNPK.D [943, 959]	K.DLEVSDPTETTLSLR.W [960, 974]	R.VSYVPITGGTPNVVTVDGSK.T [1348, 1367]	R.APVTDYLLTYESIDGR.V [1518, 1533]
Kruskal-Wallis test (p-value)		0.9738	0.8401	0.8401	0.6276
Dunn post-test analysis	High AP vs AP	ns	ns	ns	ns
	High AP vs Non-AP	ns	ns	ns	ns
	AP vs Non-AP	ns	ns	ns	ns
Spearman Correlation		*p-value*	r^2*-value*		
	***Peptide 1 and 2	<0.0001	0.9455		
	***Peptide 1 and 3	<0.0001	1.000		
	***Peptide 2 and 3	<0.0001	0.9455		

Putative ISG12(2) protein Q6IEC5		R.GSLASSIMSGEAK.S [18, 30]	K.SFGGGVPSGGTTATLQEMGAK.G [31, 51]
Kruskal-Wallis test (p-value)		0.0455	0.5232
Dunn post-test analysis	High AP vs AP	*	ns
	High AP vs Non-AP	*	ns
	AP vs Non-AP	ns	ns
Spearman Correlation		*p-value*	r^2*-value*
	Peptide 1 and 2	ns	0.4455

Hsp90 co-chaperone Cdc37 O57476		K.ELEVAEPGGGSGGGR.G [69, 83]
Kruskal-Wallis test (p-value)		0.0282
Dunn post-test analysis	High AP vs AP	*
	High AP vs Non-AP	ns
	AP vs Non-AP	Ns

Uncharacterised protein+ F1NAP7 (Mannan-binding lectin associated serine protease 2)		K.YICSDDGFWK.N [407, 416]	K.NDLVNFDNDIALIK.L [523, 536]		
Kruskal-Wallis test(p-value)		0.0406	0.0764		
Dunn post-test analysis	High AP vs AP	*	ns		
	High AP vs Non-AP	*	ns		
	AP vs Non-AP	Ns	ns		
		p-value	r^2*-value*		

Spearman Correlation					
	Peptide 1 and 2	Ns	-0.300		
Uncharacterised protein+ E1BZE1 (alpha-2-HS-glycoprotein/fetuin-A)		K.VNGQFSVLASK.C [117, 127	K.NPDVYLMLLEIGR.A [171, 183]	K.DNLAACQLLPEDQSNFGFCTAK.M [212, 233]	K.ISHNNPVASESSSSEFPSLLSAK.S [283, 305]
Kruskal-Wallis test(p-value)		0.0478	0.0455	0.0487	0.0455
Dunn post-test analysis	High AP vs AP	*	*	*	*
	High AP vs Non-AP	*	*	*	*
	AP vs Non-AP	ns	ns	ns	ns
Spearman Correlation		*p-value*	r^2*-value*		
	*Peptide 1 and 2	0.0144	0.7273		
	***Peptide 1 and 3	0.0001	0.9273		
	**Peptide 1 and 4	0.0015	0.8545		
	**Peptide 2 and 3	0.0074	0.7727		
	Peptide 2 and 4	Ns	0.5636		
	**Peptide 3 and 4	0.0086	0.7636		
Uncharacterised protein+ E1C932 (Major facilitator superfamily domain-containing protein 10)		R.SFGVFLLSR.M [138, 146]	K.EAPSDQNLQNLK.I [270, 281]		
Kruskal-Wallis test(p-value)		0.3850	0.0487		
Dunn post-test analysis	High AP vs AP	ns	*		
	High AP vs Non-AP	ns	*		
	AP vs Non-AP	ns	ns		
Spearman Correlation		*p-value*	r^2*-value*		
	Peptide 1 and 2	ns	-0.209		
Uncharacterised protein+ F1NAB7 (Complement C1r subcomponent)		K.AVDLDECDPDNAAEDDEGLR.C [115, 134]	K.SNEVDILFFTDESGYSR.G [254, 270]		
Kruskal-Wallis test(p-value)		0.4618	0.3749		
Dunn post-test analysis	High AP vs AP	ns	ns		
	High AP vs Non-AP	ns	ns		
	AP vs Non-AP	ns	ns		
Spearman Correlation		*p-value*	r^2*-value*		
	Peptide 1 and 2	Ns	0.02727		
Uncharacterised protein+ F1P580 (No close homologies)		R.LIWILK.D [63, 68]	K.DTLNLLQPVQDK.F [69, 80]	K.GYDFSFLR.R [117, 124]	
Kruskal-Wallis test (p-value)		0.9306	0.7004	0.2577	
Dunn post-test analysis	High AP vs AP	ns	ns	ns	
	High AP vs Non-AP	ns	ns	ns	
	AP vs Non-AP	ns	ns	ns	
Spearman Correlation		*p-value*	r^2*-value*		
	***Peptide 1 and 2	0.0001	0.9273		
	**Peptide 1 and 3	0.0069	0.7745		
	**Peptide 2 and 3	0.0018	0.8428		

Table 4.12: Statistical results using SIM/targeted MS2 of selected proteins proteins in three acute phase groups: HAP (n=3), AP (n=4) and NAP (n=4). (ns = no significance, *<0.05, **<0.01, ***<0.001, ****<0.0001, +Uncharacterised protein, homology given in brackets). For each protein the quantitypic peptides are listed to the right in columns, underwhich the statistical results of

the Kruskal-Wallis test are listed (p-value). The results of the Dunn post-test analysis, conducted to identify, where present, between which of the acute phase groups the significant difference lies are detailed in the rows underneath. Underneath is the result of the Spearman correlation, undertaken between the peptides for each protein across all the samples to determine whether the peptide peak areas (intensities) for each peptides, tryptically digested from the target protein correlate with each other. Asterisks to the the left of the peptide correlation indications level of significance, with p-value and r^2-value listed alongside.

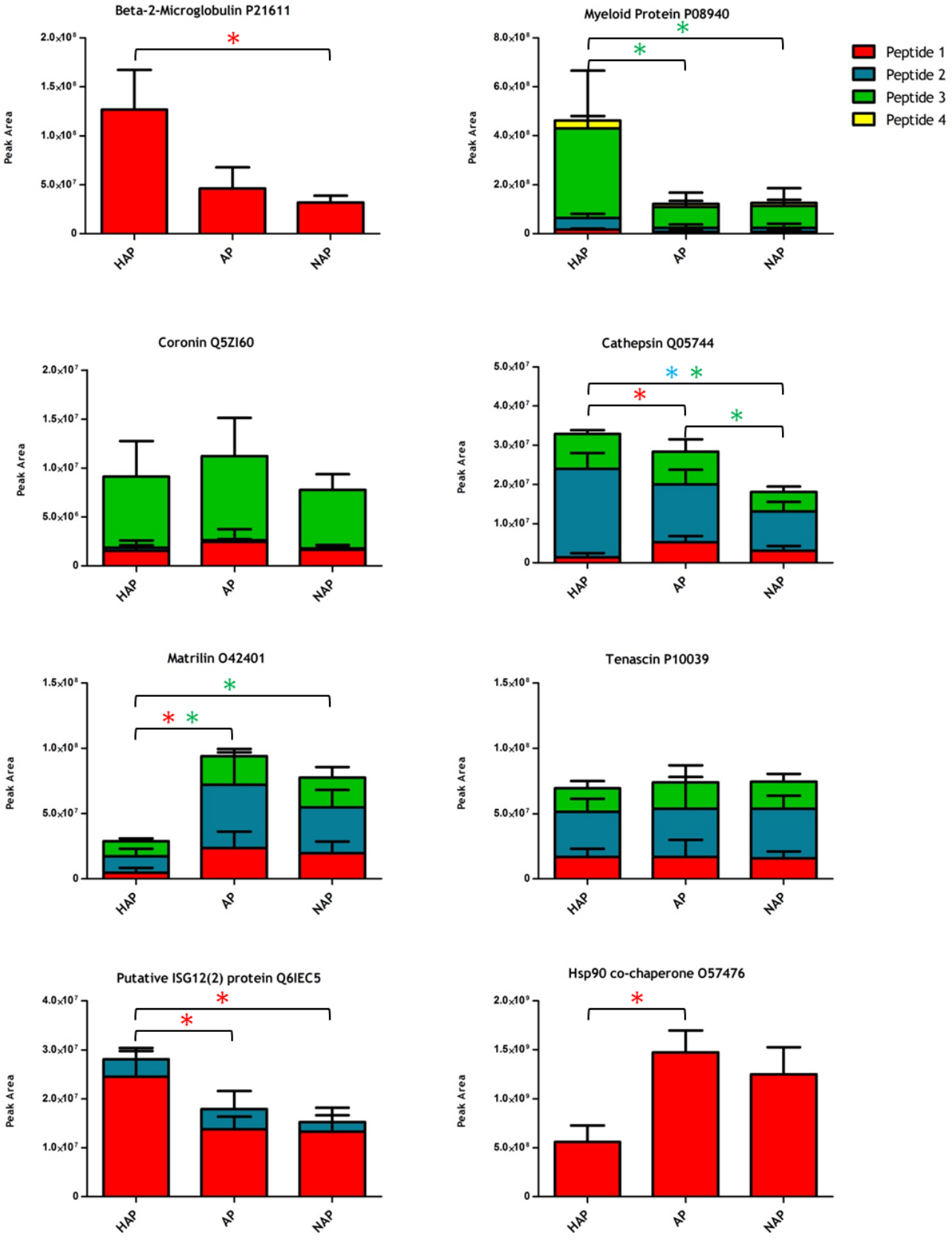

Beta-2-Microglobulin P21611
Myeloid Protein P08940
Coronin Q5ZI60
Cathepsin Q05744
Matrilin O42401
Tenascin P10039
Putative ISG12(2) protein Q6IEC5
Hsp90 co-chaperone O57476
Peak Area
HAP
AP
NAP
Peptide 1
Peptide 2
Peptide 3
Peptide 4

Figure 4.12 a

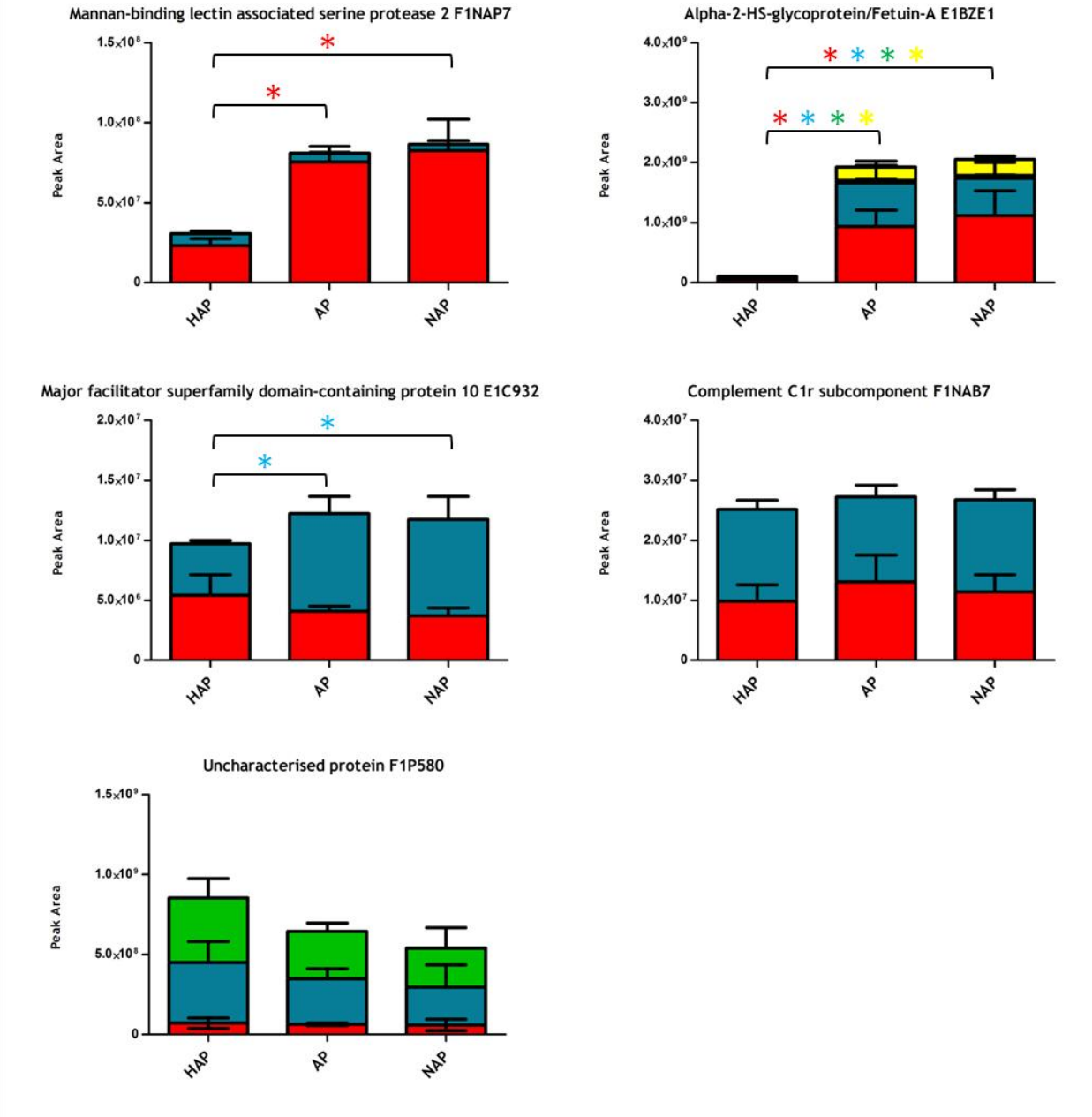


Figure 4.12b

Figure 4.12a and 4.11b: Peak area (± SD of the mean) of each targeted protein, divided within bar by peptide in three different acute phase status groups: highly acute phase (HAP) (n=3), acute phase (AP) (n=4) and non-acute phase (NAP) (n=4). Statistical analysis was undertaken using Kruskal-Wallis with Dunn post-test analysis (GRaphPad Prism v5). Significance

between groups (as detailed in Table 4.8) is indicated by asterisk *<0.05, **<0.01, ***<0.001, ****<0.0001. Colour of asterisks corresponds to same coloured peptide as indicated in legend.

For the proteins beta-2-microglobulin and HSP90 co-chaperone Cdc37, only a single peptide was successfully measured. For these two proteins the peak area of the measured peptide was found to be significantly different between the HAP and NAP and HAP and AP respectfully. Coronin, tenescin, complement C1r subcomponent and the uncharacterised protein showed no significant differences between the peak areas of the three acute phase groups, indicating that these proteins were not affected by the acute phase status of these birds when examined over individual samples as oppose to pools. Of these proteins, tenescin and the uncharacterised protein have good degree of correlation between the peak areas of the measured peptides, with tenescin particularly having all three peptides correlating with each other with p-values of <0.0001. For complement C1r component there was no significant correlation between the two peptides measured and as such one or both represent poor quantitypic peptides.

For the other selected proteins significance was found between at least two of the three acute phase groups, usually between the HAP and NAP. A number of the proteins appear to be negative biomarkers, reduced in abundance in the HAP group. Myeloid, matrilin-3, putative ISG12(2), mannan binding lectin associated serine protease-2, α-2-HS-glycoprotein/fetuin-A and major facilitator superfamily domain-containing protein 10 all show significant differences in abundance between the HAP and NAP group and the HAP and AP group, with the AP and NAP groups appearing closer in abundance. Of these proteins myeloid protein and putative ISG(12)2 are positively associated with the acute phase, the other proteins are negatively associated, being significantly less abundant in the HAP group. The protein cathepsin D, positively associated with the acute phase groups was significantly higher in both HAP and AP groups, indicating that of all the proteins targeted in this protein appears to have the most potential as a

biomarker of the acute phase, as it was significantly increased in the AP as well as the HAP group.

4.4 Discussion

Taking a quantitative proteomic approach to identifying and measuring recognised APPs allows for their confirmation as acute phase responders in chickens, allowing for the cost effective measurement of a wide number of proteins within a single study. This is an appropriate approach in this species as previous work has focused of mammalian APPs, which if transferred to chicken could result in many biomarker candidates being overlooked because they are not known as acute phase responders in mammalian species.

4.4.1 Enrichment and shotgun analysis of acute phase serum

Using protein fractionation to enrich the lower molecular weight proteins to identify SAA and CRP peptides resulted in the identification of more SAA peptides than shotgun analysis of whole serum alone. Of the SAA peptides that were identified with no miscleavages, three were identified in the whole serum and the same three together with one further peptide was identified in the fractionated serum. The fact that SAA was not identified in the NAP serum and that all three of SAA peptides were significantly higher in the HAP samples confirms previous findings that this is a major APP in chickens and as such should command the primary position in APP panels for this species.

C-reactive protein in contrast was not conclusively identified at any step of this investigation. Using a phosphorylcholine gel affinity column to isolate and purify chicken CRP was not successful, with only Apo-AI and immunoglobulin identified in the elution. Using MS to identify the proteins within the elution bands from 1DE resulted in the identification of one peptide for canine CRP when the search criteria was extended. This is likely to be a contaminant from the highly concentrated canine CRP included on the gel for comparative purposes. Furthermore when the same elution had undergone trypsin digestion and SHOTGUN proteomics no CRP or other pentraxin was identified. Using the molecular weight fractionation to enrich the lower molecular weight proteins resulted in the identification of a single CRP peptide from pool-3, the NAP pool. As a minimum of two peptides are required for the positive identification of a protein, this falls below the threshold and as such a positive identification could not be made. Searches were made on subsequent shotgun results of individual

samples and again no further CRP peptides were identified. Although it may be that peptides from trypsinisation of chicken CRP are not ideal candidates for flight in a MS, these results were generated from a range of samples: HAP, AP, NAP and enriched samples and suggest that CRP is not an APP in chickens. A CRP gene is present in the chicken genome however there is evidence of the protein only at transcript level (Uniprot, 2016). Further work is necessary to confirm the absence of CRP in the chickens.

4.4.2 Identification and measurement of acute phase proteins using data dependant single ion monitoring targeted MS/MS

The APPs SAA, Ovt, Apo-AI, Hpx, Ttn and PIT54 were all successfully identified and quantified using shotgun analysis and targeted SIM MS/MS. Although AGP was also identified it was not selected for further measurement given the widespread availability of immunoassays. This study confirmed SAA as a major positive APP in chickens, the results also validated by a commercially available immunoassay. While no significance was identified between the acute phase groups for Ovt, this is considered to be a result of the low samples numbers. Three of the four quantified peptides significantly correlated with the results of the RID assay established in Chapter 3 offering further validation of this immunoassay. Apolipoprotein AI was shown to behave as a negative APP, with significantly lower abundance detected in the HAP samples for all three peptides. Transthyretin appeared to also behave as a negative APP, although only one of the three peptides was significantly different between the three acute phase groups. Neither Hpx or PIT54 appeared to act as acute phase responders, in contrast to previous findings. Given the widespread measurement of PIT54 in a number of studies further validation of commercially available immunoassays is warranted, though again the small number of samples in each of the three groups may be an issue, particularly for minor APPs. This method identified quantotyptic peptides for six APPs, and the availability of this data enables this method to be employed in future studies of the chicken acute phase, allowing a large number of APPs to be measured without the limitations of immunoassay availability.

4.4.3 Identification and measurement of novel biomarkers of the acute phase response using data dependant single ion monitoring targeted MS/MS

Normalising and comparing the shotgun results for the HAP, AP and NAP serum samples allowed differentially abundant proteins to be identified by ranking the fold difference in abundance of each protein. This analysis was performed on pooled samples, and while this proved to be successful in identifying some proteins that went on to show significant differences between acute phase groups when analysed on individual samples; there were a number of proteins for which no significance was found when measured on individual samples.

Coronin, a highly conserved regulator of the actin cytoskeleton responsible for phagocytosis and macropinocytosis (de Hostos, 1999), tenascin, a major extracellular matrix glycoprotein that shows a very dynamic occurrence during embryonic development, in tumours and healing wounds (Spring *et al.*, 1989) and an uncharacterised protein with close homology to complement C1 whose biological functions include innate immune response, complement activation and proteolysis (Uniprot.org); were all found to show no significant differences between acute phase groups when quantified on individual samples. A further protein with no close homology, targeted because it was not present in the NAP group and showed 18 fold higher abundance in HAP compared to AP, was also found to exhibit no significant differences in abundance between acute phase groups when samples were evaluated individually. This could be an affect of small sample numbers in each group but more likely this demonstrates the need for analysing individual samples to determine whether a protein is a biomarker candidate.

For complement C1 only two peptides were identified, ideally three peptides require quantifying to make a comparative analysis on the quantity of the protein from which they were digested. For five other proteins: ß-2-microglobulin, putative ISG12(2), HSP90 co-chaperone Cdc37, mannan binding lectin associated serine protease-2 and major facilitator superfamily domain-containing protein 10 only one or two peptides were quantified, making any conclusions as to their behaviour between the acute phase groups difficult. Analysis of the measured peptides does however provide an initial insight into the behaviour of the protein during an APR.

For ß-2-microglobulin a single quantotypic peptide was identified and measured, revealing a significantly higher abundance in the HAP. ß-2-microglobulin is a light chain that is non-covalently bound to the heavy chain of major histocompatibility complex (MHC) class I, the presence of which is an important prerequisite for the binding of small ligands by MHC class I molecules and maintaining complex stability (Hee *et al.*, 2012). The kidneys play a key role in the catabolism of ß-2-microglobulin and blood and urine levels are useful indicators of renal function (Cooper & Plesner, 1980). Many tumours can be associated with an elevated serum ß-2-microglobulin but it is most widely used as a tumour biomarker in lymphoid malignancies (Cooper & Plesner, 1980). Given the significance identified in this study and the wide use as a biomarker in human medicine, the inclusion of its quantotypic peptide in future studies would be recommended and further evidence of its potentiality as a biomarker in chickens may prompt development of immunoassay. Interferon stimulated gene 12 (ISG12) expression sensitizes cells to apoptotic stimuli and influences the innate immune responses of interferons (Cheriyath *et al.*, 2011). ISG12 gene expression is unregulated during infectious disease, inflammatory bowel disease and cancer (Uhrin *et al.*, 2013). In this study two putative chicken ISG12(2) peptides were quantified and one was found to have significantly higher abundance in the HAP group than AP and NAP. For HSP90 co-chaperone Cdc37, only a single peptide was identified and measured and this was significantly lower in abundance in the HAP. Cdc37 (cell division cycle protein 37) is an essential co-chaperone of Hsp90 (90 kDa heat-shock protein), a molecular chaperone that manages protein folding and quality control in the cell environment (Grover *et al.*, 2011). In chickens mannan binding lectin (MBL) is described as a mild positive APP increasing two-fold with infectious bronchitis virus, ILV and IBDV (Laursen & Nielsen, 2000). Mannan binding lectin is part of the lectin pathway an important component of innate immunity providing a first line of defence during the lag phase that precedes the onset of an adaptive response (Degn *et al.*, 2012; Lynch *et al.*, 2005). The lectin pathway is an antibody independent route of complement activation (Lynch *et al.*, 2005). This pathway is activated by multi-molecular complexes that recognize and bind to microbial polysaccharides. These complexes comprise a multimeric carbohydrate recognition subunit either MBL or ficolin and in chickens, the MBL-associated serine proteases-2 (Lynch *et al.*, 2005). In this study, MBL was not identified in

the shotgun analysis of any of the acute phase pools or individual samples. However the MBL-associated serine proteases-2 was found and two peptides were measured. One peptide showed significantly lower abundance in the HAP compared to both the AP and NAP, indicating it is negatively associated with the APR. Consideration must be given to the fact that only a single peptide is being evaluated as opposed to the recommended three, however this is an interesting results and its quantification in future studies, particularly alongside the APP MBL would provide useful insight into the avian lectin pathway. Major facilitator superfamily domain-containing protein 10 is a member of the major facilitator superfamily (MFS) the largest groups of secondary active transporters that play a pivotal role in multiple physiological processes (Yan, 2013). Only one of the two peptides measured from this protein was found to be significant between acute phase groups, with lower abundance in the HAP group.

The protein that appears to show the most potential as a biomarker of the APR is cathepsin D. The major function of cathepsin D is an aspartate protease involved in intracellular catabolism and is found within the acidic fraction of the lysosomal compartment (Dean, 1975; Fusek & Vetvicka, 2005). It is also involved in the processing of antigens, hormones, and neuropeptides as well as tissue remodelling and apoptosis. Cathepsin D serves as an independent prognostic factor in many types of cancers as pro-cathepsin D has the ability to stimulate growth and cancer cell proliferation to form metastases (Fusek & Vetvicka, 2005). All three peptides showed significant differences between the acute phase groups, with increased abundance of all three cathepsin D peptides positively associated with the APR. Furthermore cathepsin D was significantly higher in the AP compared to the NAP, and as such appears to be a more sensitive protein showing significant increases in this middle acute phase group. Other proteins showed significance between the HAP and NAP or HAP and AP groups only. Matrilin-3 is an extracellular matrix (ECM) protein that connects matrix components in the cartilage to form macromolecular networks (Shahzad *et al.*, 2014). In humans a strong correlation exists between enhanced matrilin-3 protein expression with the extent of tissue damage in cartilage of osteoarthritis patients, suggesting that matrilin-3 expression is essential for maintenance of the cartilage ECM microenvironment (Shahzad *et al.*, 2014). In chickens matrilin-3 is mainly expressed in growth plates with reduced expressions of matrilin-3

mRNA and protein during TD in chickens (Shahzad *et al.*, 2014). In humans matrilin-3 can be measured in synovial fluid and serum and its measurement in serum can be used as a biomarker for osteoarthritis (Vincourt *et al.*, 2012). In this study three matrilin-3 peptides were identified and measured and two of the three were found to be significantly lower in the HAP. Myeloid protein-1 precursor is expressed specifically by cells of hematopoietic origin. It is also abundant in osteoclasts which are derived from myeloid precursors and it is postulated to be important in bone remodelling (Falany *et al.*, 2001). In birds myeloid protein-1 has also been found to be a novel chemoattractant factor for heterophils and has been associated with the host response to *Salmonella* infection (Sadeyen *et al.*, 2006). One out of the four quantified myeloid protein-1 peptides showed a significant difference between acute phase groups, being significantly higher in the HAP group.

Fetuin, also called α-2-glycoprotein, is a multifunctional plasma protein and a member of the cystatin superfamily of protease inhibitors identified in humans, rodents, sheep, pigs and cows (Gangneux *et al.*, 2003; Dabrowska *et al.*, 2015). The name fetuin (or fetuin-A in humans and rats where a paralog called fetuin-B also exists) has been suggested as the universal nomenclature for this protein in every species (Olivier *et al.*, 2000; Gangneux *et al.*, 2003). During foetal development fetuin is abundantly synthesized by multiple tissues, in adults it is secreted predominantly by the liver (>95%) (Dabrowska *et al.*, 2015). Fetuin-A is a key protein in several metabolic pathways and accumulates in bones and teeth as a major fraction of non-collagenous bone protein. It is involved in osteogenesis and bone resorption, this function ascribed to the ability of fetuin-A to regulate calcium metabolism and growth factor-β activity required for bone mineralization (Olivier *et al.*, 2000; Gangneux *et al.*, 2003; Dabrowska *et al.*, 2015). Fetuin-A is an anti-inflammatory mediator that participates in macrophage deactivation preventing the overproduction of pro-inflammatory cytokines (Gangneux *et al.*, 2003). It appears to be a key partner in the recovery step of an APR to systemic inflammation (Olivier *et al.*, 2000). The behaviour of fetuin-A during an APR varies between species: in humans, rats and guinea pigs fetuin(-A) is reported as a negative APPs, whereas in mice and cattle it is described as a positive APP (Olivier *et al.*, 2000). In cattle fetuin decreases by 30 % in pregnancy but increases up to 10-fold in some trauma cases (Dziegielewska

et al., 1992). Given its crucial role in the pathogenesis of type 2 diabetes mellitus, obesity, nonalcoholic fatty liver disease, dyslipidemia, atherosclerosis and cardiovascular disease in humans fetuin-A is regarded as a useful biomarker in clinical practice (Dabrowska *et al.*, 2015). In chickens feutin has been identified in bone (Gotoh *et al.*, 2002) though there is no reference in the literature to its role during the APR. In this study an uncharacterised protein with close homology to alpha-2-HS-glycoprotein/fetuin-A was identified. Four peptides were quantotypic and all four peptides had significantly lower abundance in the HAP group. The abundance of all peptides were similar and insignificant between the AP and NAP, but the levels detected in the HAP were dramatically lower. In the pooled samples the abundance in the HAP pool was 44 and 56 fold lower than the AP and NAP pools respectfully. This dramatic difference is highly suggestive of this being a negative APP in chickens.

It is notable that matrilin-3 (a protein of the ECM in cartilage), myeloid protein-1 (associated with bone remodelling) and fetuin (involved in the regulation of osteogenesis and calcium metabolism) are all significantly different in the HAP group. This suggests that in the HAP group further changes associated with bone homestasis are occurring. Mireles *et al.*, (2005) found that inducing an APR by *E. coli* LPS injection in chickens caused marked changes in bone metabolism, evidenced by a loss of tibia breaking strength. Bone homeostasis was severely disturbed as a result of the inflammatory response possibly as a result of changes in the ECM or in the mineral matrix due to changes in calcium or phosphorus metabolism during the APR (Mireles *et al.*, 2005). In mammals, LPS activates osteoclasts and causes bone resorption via pro-inflammatory cytokines (Roux and Orcel, 2000). The significant differential expression of proteins associated with bone and cartilage metabolism in chickens that are highly acute phase should prompt further studies into the effects of the APR on bone metabolism, as the resultant changes in the associated plasma biomarkers may elude to more information on both the APR and skeletal health in chickens.

4.4.4 Identifying acute phase biomarkers – future perspectives

While this study was successful in identifying a number of chicken APPs and novel biomarkers of the APR, it highlighted a number of areas to be considered in future studies. While the initial analysis concentrated on identifying proteins with the largest fold difference between the three pools, this was presumptive as it was only identifying and ranking proteins that were found in all three pools. There were a number of proteins, including SAA that were absent in the NAP pool yet highly expressed in the AP and HAP pools, and as such excellent candidates for further study and subsequent analysis took this into consideration. While many studies use well established models to induce an APR, this study incorporated samples from farm culls that were classified according to the serum concentration of the APP AGP. This is a well established APP and the most widely studied APP in chickens to date and this resulted in three clearly defined acute phase groups. This also had the potential however to shape the subsequent findings, particularly as the NAP, while having normal AGP concentrations were culled for stunting or lameness and as such could have underlying non-inflammatory pathologies. Furthermore the HAP group were a viraemic group with extremely high serum AGP concentrations reflecting this particularly sick group of birds. While surveying for biomarkers as this study has done, these may not be relevant issues, further studies may benefit from utilising samples from more controlled and established models of APR.

Although identifying biomarkers such as APPs in very sick birds or those challenged with LPS in trials provide information on how APPs respond to pathogens; identifying and measuring more ‘subtle’ biomarkers of the acute phase may allow early detection of disease and ill health in a flock and separate the healthy and unaffected from the (apparently) healthy and mildly/early infected. Using untargeted quantitative shotgun method as employed in this study is an excellent way of comparing groups and individuals by identifying all the proteins in plasma that show differential expression. In this study many proteins were found to be differentially expressed in the HAP group when compared to the AP and NAP groups. If the study was repeated with birds challenged with LPS, again major differences in the plasma proteome would likely be detected. Future studies should aim to identify protein biomarkers expressed in the early and mild APR to allow for the early detection of disease,

allow more of distinction to be made between birds during low levels of infection and as such be of greater diagnostic value.

Chapter 5

Biomarkers of intestinal health, satiety and the acute phase response in chickens challenged with re-used litter

5.1 Introduction

The consequences of intestinal disease in production animals are wide ranging, affecting welfare, productivity and potentially human health. Identifying areas where the application of proteomics and immunoassays could serve to improve understanding of disease processes and be useful for diagnosis and monitoring flock health are welcomed. In poultry production intestinal health and function is paramount to achieving efficient feed utilisation and growth. Maintaining a high level of enteric health within a flock allows for the profitable production in a high welfare environment, of meat products that are safe for the consumer.

This Chapter investigates, in more depth, areas of interest highlighted during previous studies. In Chapter 2, the mapping of the 2D proteome of chicken plasma revealed a distinct spot that varied in size between samples (Figure 2.8, spot 18). This spot was identified as apolipoprotein AIV (apo-AIV), an intestinally derived antioxidant protein that associates with HDL and functions in reverse cholesterol transport, lipid transport and lipoprotein metabolism (Franceschini, 2003; Vowinkel *et al.*, 2004; Navarro *et al.*, 2005). Apolipoprotein AIV has also been shown to function as a satiety protein to control food intake and gastric function in mammals (Tso & Liu, 2004; Vowinkel *et al.*, 2004). Apolipoprotein AIV is associated with the APR, being an endogenous anti-inflammatory protein with anti-atherogenic properties (Vowinkel *et al.*, 2004). These findings make apo-AIV both a relevant and interesting target for study in chickens.

Previous work identifying differentially expressed jejunal proteins in chickens receiving a microbial challenge in the form of re-used poultry litter, revealed the intestinal protein villin to be differentially expressed over time between challenged and control groups, decreasing in the challenged birds significantly over time (O'Reilly, 2013). Figure 5.1, taken from this study, details the decrease in expression of villin over time. These results, together with the measurement of serum villin in humans and identification of villin as a biomarker for intestinal dysfunction (Wang *et al.*, 2008) make villin an appropriate target for further research. Gelsolin, a member of the gelsolin super family of actin binding proteins to which villin belongs, was also selected for measurement using quantitative proteomics.

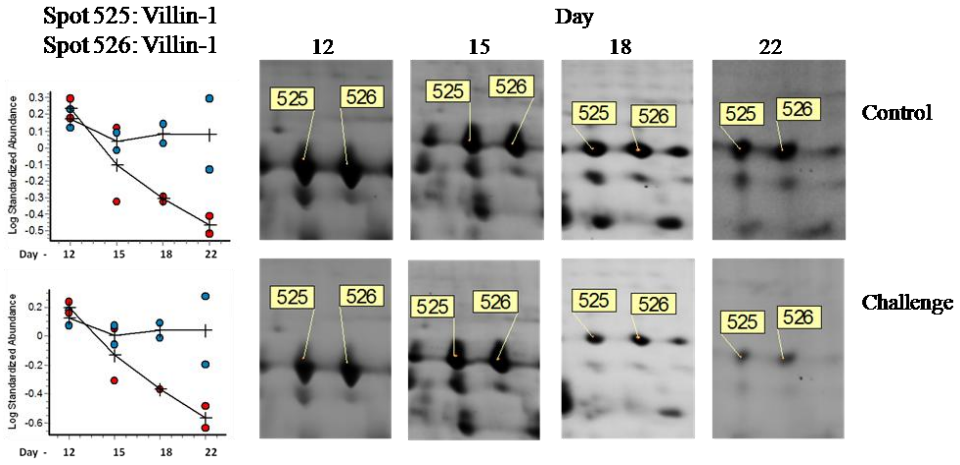


Figure 5.1: The decrease in the abundance of spots 525 and 526, both identified as villin, from day 12 (pre-challenge) to day 22, in birds challenged with re-used poultry litter (O'Reilly, 2013). Proteins were seperated and quantified using DiGE.

5.1.1 Apolipoprotein AIV

In humans and rats apo-AIV is synthesised in the proximal small intestine, the liver and the hypothalamus and is present in plasma, interstitial fluid and lymph (Tso, *et al.* 1999; Navarro *et al.*, 2004; Pardina *et al.*, 2009). In chickens apo-AIV synthesis occurs in the intestine and liver, with the intestine being the primarily site of synthesis, the highest level of expression seen in the proximal intestine with only minor expression in the ileum and rectum. No apo-AIV mRNA has been detected in other organs investigated, including the brain (Steinmetz *et al.*, 1998). Small intestinal apo-AIV synthesis is stimulated by active lipid absorption with triglyceride feeding an potent stimulus for intestinal secretion of apo-AIV (Tso *et al.*, 1999). Following synthesis in the enterocytes, apo-AIV enters the circulation on the surface of nascent chylomirons but thereafter dissociates from their surface and circulates primarily as a lipid-free protein (Weinberg *et al.*, 2000; Weinberg, 2002). An important difference between mammalian and avian apo-AIV is that in birds apo-AIV it is not delivered to a lymphatic system but is secreted into the portal vein as a so-called 'porto-microns' which are subject to rapid uptake by the liver (Steinmetz & Schneider, 1999).

5.1.1.1 Apolipoprotein AIV structure

Apolipoprotein AIV is a 46kDa protein that has several distinctive structural features. Like all plasma apolipoproteins it contains multiple amphipathic α-helices, in apo-AIV however, these are very hydrophilic and cannot deeply penetrate lipid surfaces resulting in weak lipid binding affinity (Weinberg, 2002). In contrast the 38-kDa avian homolog of mammalian apo-AIV is a much more hydrophobic protein with longer α-helical hydrophobic domains and shorter hydrophilic domains and is predicted to contain multiple amphipathic α-helical domains and therefore binds lipid more readily (Weinberg *et al.*, 2000).

Sequence analysis reveal that apo-AIV is the most recently evolved apolipoprotein, probably originating from the intra-exonic duplication of the apo-AI gene, 270–307 million years ago. The C terminus of human apo-AIV contains a unique and highly conserved region that is the site of genetic polymorphisms that affect apo-AIV structure, plasma lipid levels, and the response to diet in mammals (Weinberg *et al.*, 2000). The primary structure of chicken apo-AIV contains 347 amino acids and displays 57% sequence identity with human apo-AIV. The chicken apo-AIV protein does not appear to be significantly glycosylated and the isoelectric point is 4.65, considerably more acidic than human apo-AIV (Steinmetz & Schneider, 1999). Avian apo-AIV lacks the highly conserved mammalian C-terminal region suggesting that chicken apo-AIV could be the molecular ancestor of mammalian apo-AIV and that the mammalian C-terminal domain may be a structural adaptation that evolved to confer a new physiologic function to this apolipoprotein (Weinberg *et al.*, 2000).

5.1.1.2 Apolipoprotein AIV function

5.1.1.2.1 Lipoprotein metabolism and reverse cholesterol transport

Evidence suggests that one of the primary roles of apo-AIV is the regulation of intestinal lipid absorption and chylomicron assembly (Weinberg *et al.*, 2000). During subsequent plasma passage and metabolism of lymph chylomicrons about 25% of apo-AIV is transferred to HDL and the remaining 75% is found in the lipoprotein-free fraction of plasma (Vowinkel *et al.*, 2004). Apolipoprotein AIV plays an important role in reverse cholesterol transport by stimulating lecithin:cholesterol acyltransferase activity which causes apo-AIV to associate

with HDL in a reaction that causes depletion of HDL surface lipids and stabilizes HDL (Navarro *et al.*, 2005).

5.1.1.2.2 Satiety

Apolipoprotein AIV has been proposed to physiologically control food intake and this inhibitory effect is centrally mediated (Tso & Liu, 2004). It is synthesised in the hypothalamus and the hypothalamic apo-AIV level is reduced by food deprivation and restored by lipid feeding (Tso & Liu, 2004). In rodent models, apo-AIV administered either intravenously or into the intra-cerebroventricular space suppresses food intake in a dose dependant manor, the potency of intra-cerebroventricular administration fifty times greater than the intravenous administration (Fujimoto *et al.*, 1992; Fujimoto & Fukagawa, 1993). Conversely administration of apo-AIV antiserum into the intracerebroventricular space causes increased feeding and decreases the hypothalamic apo A-IV mRNA level, implying that feeding is normally limited by endogenous apo-AIV (Tso & Liu, 2004).

The stimulation of intestinal synthesis and secretion of apo-AIV by lipid absorption is rapid (Tso & Liu, 2004). In rats a gastric bolus of lipid causes a significant increase of plasma apo-AIV within 15 minutes with the increment remaining significant until 30 minutes after the meal, indicating that the increase in plasma levels of apo-AIV produced were sufficiently quick and large enough to produce satiety (Rodriguez, *et al*. 1997) and that apo-AIV is capable of short-term regulation of food intake. Evidence also suggests apo-AIV's involvement in the long-term regulation of food intake and body weight. Chronic ingestion of high fat blunts the hypothalamic apo-AIV response to lipid feeding and may therefore explain why chronic intake of high fat predisposes animals and humans to obesity (Tso & Liu, 2004).

In humans genetic polymorphisms of apo-AIV are well documented and may be a factor that mediates the heterogeneous response of plasma lipids to diet in humans. Only two of the reported polymorphisms however demonstrate altered biophysical properties that include either an increased or decreased postprandial hypertriglyceridemia, LDL response to dietary cholesterol in the setting of a

moderate fat intake, HDL response to changes in total dietary fat content and higher or lower body mass and adiposity (Weinberg, 2002).

In veterinary species apo-AIV has not been well documented, the pig however is the notable exception. Pigs are regarded as one of the best models for human lipid metabolism because their lipoprotein profile is similar to that of humans (Navarro *et al.*, 2004) and as such it is the veterinary species in which apo-AIV has been most widely studied, albeit as a inflammation model.

It is only the cow and dog in which apo-AIV has been studied in relation to feeding and satiety. In cows, following the successful development of an ELISA, serum apo-AIV was found to be significantly higher in lactating cows compared to non-lactating with average apo-AIV concentrations of 102 and 64mg/L respectfully and apo-AIV concentrations of fasted calves increased significantly once fed (Takahashi *et al.*, 2004). In dogs cholesterol feeding was found to increase plasma apo-AIV three fold (Sloop *et al.*, 1983).

5.1.1.2.3 Apolipoprotein AIV and the acute phase response

Besides changes in APP, the APR is also associated with systemic alterations in lipids and lipoproteins. During infection and inflammation serum triglyceride levels increase and small dense LDL appear and HDL cholesterol levels decrease (Khovidhunkit *et al.*, 2004). Apolipoprotein AIV has antioxidant and anti-inflammatory activity (Vowinkel *et al.*, 2004) and is a potent endogenous inhibitor of lipid oxidation (Qin *et al.*, 1998). In a murine model of colitis, apo-AIV significantly and specifically delays the onset and reduces the severity and extent of inflammation. Apolipoprotein AIV significantly inhibits leukocyte and platelet adhesive interactions (Vowinkel *et al.*, 2004). Over expression of human or mouse apo-AIV in transgenic mice protects against the formation of diet-induced aortic lesions (Navarro *et al.*, 2004). In mice injected with endotoxin, increased levels of apo-AIV in acute phase HDL and apo-AIV hepatic mRNA are detected both of which indicate that this protein is a positive APP (Khovidhunkit *et al.*, 2004). Clinically it has been observed that in horses apo-AIV significantly increases in horses with both acute and chronic laminitis, a debilitating disease of the equine foot associated with insulin resistance, obesity and gastrointestinal inflammation (Steelman & Chowdhary, 2012).

In contrast, rat models of rheumatoid arthritis, that demonstrate changes to serum proteins similar to that seen during an APR, show a down regulation of apo-AIV (Eberini *et al.*, 2000) and during a turpentine oil induced APR in pigs, a decrease in hepatic apo-AIV mRNA expression is observed, though this does not correlate with the plasma protein levels. The distribution of plasma apo-AIV experiences a shift from HDL to larger particles. The well-known HDL decrease found in the APRs appears, in the pig, due to the decreased expression of apo-AI and the enlargement of the apo-AIV containing HDL (Navarro *et al.*, 2005).

There is evidence that the protective anti-inflammatory effects of apo-AIV are independent of the effects of this protein on lipoprotein metabolism and food intake, a study finding that the over expression of apo-AIV in transgenic mice whilst conferring increased protection against atherosclerosis, did not affect their feeding behaviour (Qin *et al.*, 1998). Figure 5.2 summarise the functions and the sites of synthesis of apo-AIV.

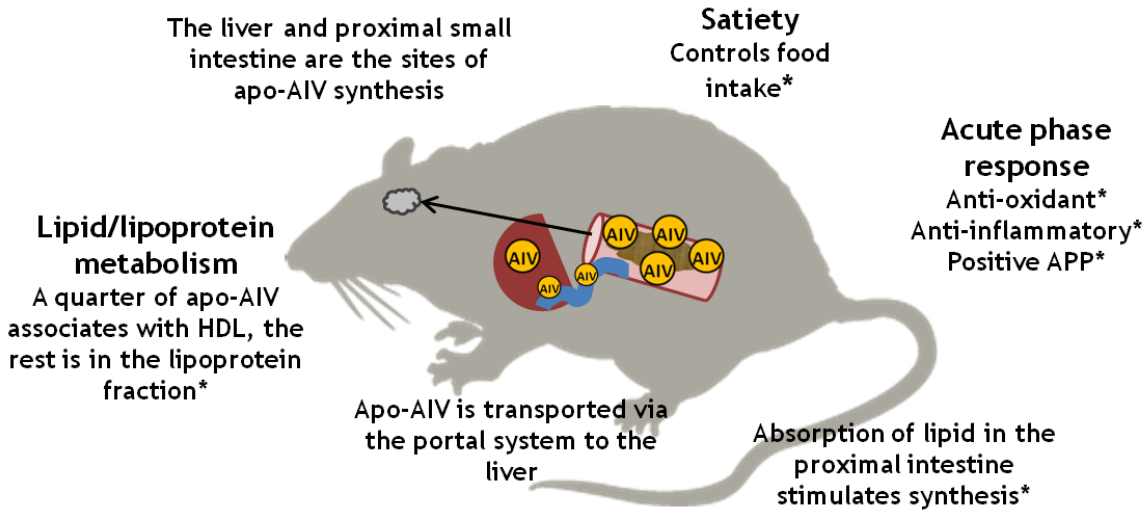


Figure 5.2: The sites of synthesis and functions of apo-AIV (*described for mammals, not demonstrated in the chicken).

5.1.2 Villin

In cells the actin cytoskeleton plays a central role in many fundamental cellular processes involving the generation of force and facilitation of movement. The assembly and three-dimensional organisation of actin filaments is controlled through interactions with a large number of functionally distinct actin binding proteins (Li *et al.*, 2012). In the intestine, epithelial cells have a brush border composed of numerous membrane extensions called microvilli, fingerlike extensions that increase the absorptive function of these specialized cells. The core of each intestinal microvillus is a rigid bundle of 20–30 parallel actin filaments extending from the microvillus tip down into the cell cortex (Athman *et al.*, 2002). Villin is the actin binding protein expressed in the brush border of the intestinal epithelium. It is also found to be highly expressed in the gall bladder and kidney (Athman *et al.*, 2002; Nag *et al.*, 2013). Villin belongs to gelsolin superfamily of actin-binding proteins which includes gelsolin, severin, fragmin, adseverin/scinderin and actin crosslinking proteins such as dematin and supervillin (Khurana & George, 2008)

5.1.2.1 Villin structure and function

Villin was first isolated from chicken intestinal epithelial cells and later from mammalian species. It has a globular protein with a molecular mass of 92.5 kDa and occurs in monomeric form, present as two eqimolar variants with isoelctric points 6.2 and 6.3 (Alicea & Mooseker, 1988). Villin can be highly phosphorylated with ten tyrosine phosphorylation sites identified in human villin (Khurana & George, 2008). All actin binding proteins in the gelsolin superfamily require calcium to function with two major calcium-sensitive sites present in villin that induce conformational changes (Khurana & George, 2008). Villin however, unlike the rest of the gelsolin superfamily, requires micromolar to millimolar levels of calcium to function as tyrosine phosphorylation lowers the threshold to the nanomolar range, suggesting that tyrosine phosphorylation may be the primary mode of regulation of villin activity under physiological conditions (Nag *et al.*, 2013).

As a versatile actin modifying protein able to both polymerize and depolymerize actin filaments and cap, sever, nucleate and bundle actin filaments (Li *et al.*, 2012), villin is able to regulate epithelial cell morphology, actin reorganisation, apoptosis, cell motility and wound healing of intestinal enterocytes (Wang *et al.*,

2008; Nag *et al.*, 2013). This is necessary for the intestinal epithelium to function as a physiological and structural barrier as intestinal epithelial cells have a high rate of cell turnover accompanied by an equally high rate of apoptosis (Li *et al.*, 2012).

5.1.2.2 Villin as a biomarker

Normal apoptosis is essential for the hierarchical organisation of the intestinal epithelium and apoptosis is a key regulator of the very high cellular turnover rate seen in the gastrointestinal epithelium (Li *et al.*, 2012; Wang *et al.*, 2012). Abnormalities associated with apoptosis in the epithelium have been linked to most major gastrointestinal disorders, including ischemia reperfusion injury, inflammatory bowel disease, necrotising enterocolitis, coeliac disease, gastrointestinal infections and colorectal cancer (Wang *et al.*, 2012). Increased apoptosis in the intestinal epithelium contributes to the tissue damage and the severity of colonic inflammatory response (Li *et al.*, 2012). A decrease in the levels of villin expression in enterocytes has been noted in several inflammatory diseases of the gastrointestinal tract including Crohn's disease and ulcerative colitis (Wang *et al.*, 2008; Li *et al.*, 2012).

As well as changes to villin expression in the intestine, the presence of villin in the serum is also indicative of the pathological state of the gastrointestinal tract, with significant associations between serum villin and stage, severity and type of gastrointestinal disease found (Dudouet *et al.*, 1990). Because villin expression is maintained in neoplasic tissues, villin is also a useful marker for primary tumors or metastases deriving from tissues that normally express villin. Its presence in the blood of patients presenting colorectal carcinomas makes it a diagnostic adjunct for the detection of these cancers (Athman *et al.*, 2002).

5.1.3 Gelsolin

Gelsolin is a member of the gelsolin superfamily of actin binding proteins all of which are calcium dependant multi-domain regulators of the actin cytoskeleton (Nag *et al.*, 2013). In mammals villin shares 50% sequence homology with gelsolin and has a similar proteolytic cleavage pattern (Nag *et al.*, 2013). In chickens gelsolin and villin share 40.3% sequence homology (Uniprot.org). Gelsolin is a highly abundant, calcium dependent actin binding protein and there is increasing

evidence that gelsolin is also a multifunctional regulator of cell metabolism that involves multiple mechanisms independent of its actin regulatory functions (Li *et al.*, 2012). Gelsolin is the most widely studied actin regulatory protein, however the roles of gelsolin are disputed in terms of its involvement in contrasting activities such as oncogenic, anti-oncogenic, pro-apoptotic and anti-apoptotic (Khurana & George, 2008). While both members of the same functional family, gelsolin unlike villin is not tissue specific. Gelsolin exists in two forms: a plasma form and a cytoplasmic form and cytoplasmic gelsolin is ubiquitously expressed in cells and tissues (Li *et al.*, 2012).

Plasma gelsolin depolymerises and sequesters actin released into the vasculature as a result of lysis of injured or dead cells and tissues, averting pathological blockages of the microvasculature (Li *et al.*, 2012; Nag *et al.*, 2013). This scavenging role of gelsolin involves the binding and inactivation of bioactive inflammatory mediators. Gelsolin plays a protective role against inflammation and can enhance or inhibit apoptosis depending on the nature of the pathological conditions, the identity of the cell types, and the specific tissues that are involved (Li *et al.*, 2012).

5.1.3.1 Gelsolin as a biomarker

Gelsolin has a variety of roles in the pathology of many diseases and as such is reported in humans to have biomarker and therapeutic target potential (Li *et al.*, 2012; Nag *et al.*, 2013). Increased plasma gelsolin expression has been detected in acute liver failure, myocardial infarction, septic shock, muscle necrosis and acute respiratory distress syndrome. Gelsolin is observed to be down regulated in most cancers, except a small sub-set of lung cancers and in the transition from non-invasive to invasive tumours. The levels of gelsolin in a cancer setting may give an index of metastatic potential and is thought to be an independent marker for tumour recurrence and progression in urothelial tumors, particularly for high-grade variants. Gelsolin may also be used as a biomarker in cardiovascular disease, with its abundance an indicator of chronic heart failure (Li *et al.*, 2012). Gelsolin is involved in a wide range of cellular processes, influencing actin dynamics in multiple and sometimes contradictory ways. As such, while gelsolin is regarded as an assayable peripheral blood biomarker for

several disease conditions, the changes in plasma gelsolin concentrations are regarded as context sensitive (Li *et al.*, 2012; Nag *et al.*, 2013).

5.1.4 Aims of this study

Apolipoprotein AIV is a multifunctional protein, reported to be an acute phase reactant in mammals. No studies characterising apo-AIV during an APR in chickens have been undertaken and apo-AIV does not appear to have been targeted in poultry nutritional or feed behaviour studies. The development of an ELISA to measure plasma/serum apo-AIV in chickens would therefore be of great value to many poultry research areas and develop a better understanding of how this lipoprotein functions in the chicken. It was hypothesised that, once quantifiable and measured, apo-AIV would be affected by the addition of re-used litter to challenged pens. Furthermore it was also hypothesied that apo-AIV would also change during an APR.

Using re-used litter provides a microbial challenge to broilers that is representative of the commercial growing environment. Re-used litter changes the intestinal microbiota and its increased microbial complexity acts as an immunological challenge to the chicks and negatively affect growth rates (Torok, *et al.*, 2009; Cressman *et al.*, 2010; Lee *et al.*, 2011). Acute phase proteins, using both immunoassays and quantitative proteomics, will be measured to determine whether the addition of re-used litter causes an APR, the hypothesis being that the litter could evoke an immune response sufficient enough to increase serum concentrations of APPs. Having already established that the introduction of re-used poultry litter negatively affected growth rates and causes differential expression of a number of intestinal proteins, including villin (O'Reilly, 2013), it was the aim of this Chapter to validate the change in villin over time in the intestinal lysate and investigate whether villin could be a biomarker for intestinal health in the broiler chicken by identifying, and if possible, quantifying this protein in the plasma of chickens using quantitative proteomics and immunoassay methods. The hypothesis being that villin, having been demonstrated to decrease in the intestinal proteome of challenged birds, would also show a similar change in the serum of these birds.

5.2 Methods

5.2.1 Intestinal challenge study

This study was undertaken at the Scotland's Rural College (SRUC) in 2012 for an M.Sc. project investigating the intestinal proteome of broiler chickens (O'Reilly, 2013) in collaboration with Professor Nick Sparks. During this experiment, blood was also recovered for the purposes of studying the APR. The experimental model was approved by the SRUC animal experimentation committee and SRUC standard operating procedures (SOPs) were employed throughout the experiment to ensure the welfare of the birds was maintained. At day-old, 120 male Ross 308 broiler chicks were randomly divided into two groups: control and challenged. Birds were divided into 6 replicates per group, housed in individual pens containing 10 birds per pen in an environmentally controlled room (standard temperature profile). Chicks were fed a commercial starter ration until 10 days of age after which they were moved onto a grower ration for the remainder of the study. On day 12 litter from a commercial unit housing broilers aged 35 days was recovered from the top layer, manually broken down and 1 kg was added to each of the challenge pens. An aliquot of the challenge litter was submitted to the Avian Science and Research Centre (ASRC) microbiology laboratory's (SRUC, Auchencruvie, UK) for microbial analysis. On day 12 and days 15, 18 and 22 (3, 6 and 10 days post addition of the reused litter) the chicks in each pen were bulk-weighed and one bird from each pen was randomly selected and following cervical dislocation blood was recovered via intra-cardiac puncture into lithium containers. Following centrifugation, plasma was removed, aliquoted and stored at -80˚C until use. Figure 5.3 gives an overview of experimental design.

5.2.2 Measurement of acute phase proteins

5.2.2.1 Immunoassays

The APPs SAA and Cp were measured using commercially available ELISAs (3400-7 (SAA), 2610-3 (Cp) Life Diagnostics, Stoke-on-Trent, UK). Both ELISA's utilise chicken specific antibodies and although used according to the manufacturer's instructions, plasma was diluted 20 fold and 40,000 fold with diluent for the SAA

and Cp ELISAs respectfully, to obtain results that were measureable on the standard curve. For the SAA ELISA a smaller volume of plasma was also used with resultant adjustments to diluent volumes, to accommodate the small volumes of plasma available. For Ovt the RID method described in Chapter 3 was used. Mann-Whitney non-parametric tests (GraphPad Prism v.5) were used at each time point to determine whether the plasma concentrations of these APPs were significantly different between challenged and control groups. A Krusal-Wallis test was used to identify significance over the four time points for the challenge and control samples separately.

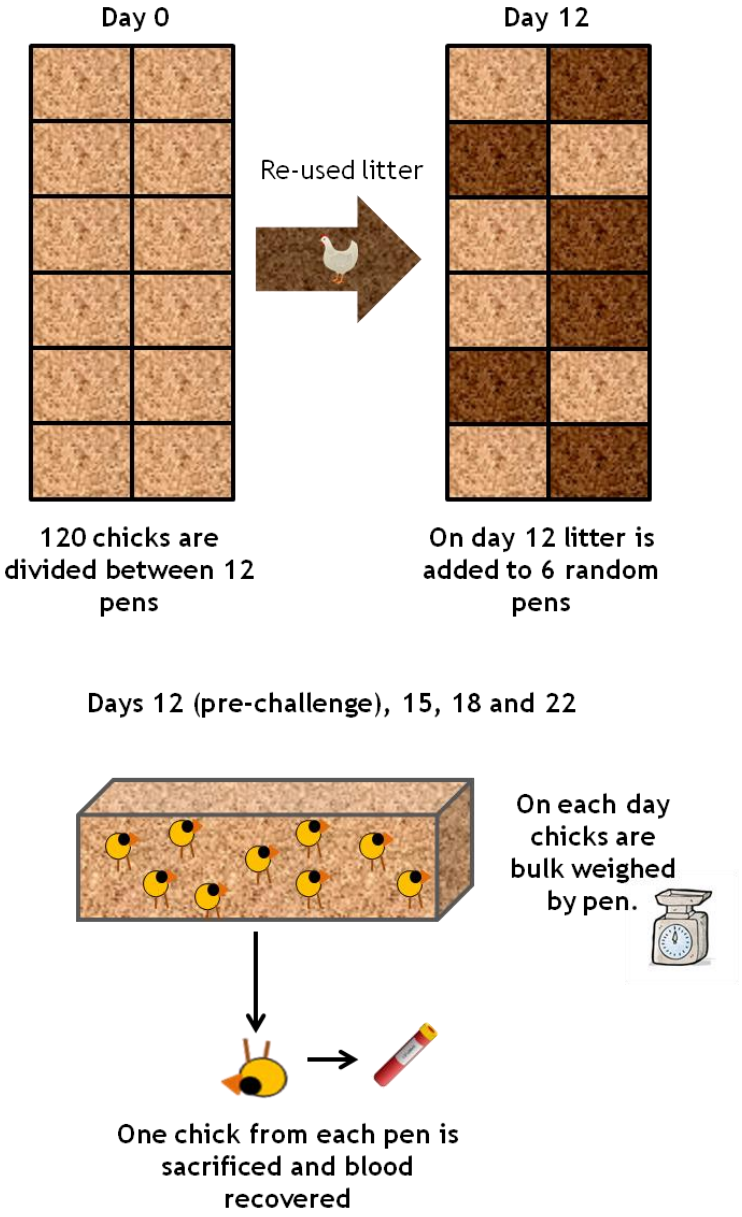


Figure 5.3: Overview of intestinal challenge experimental design.

5.2.2.2 Quantitative proteomics

The APPs SAA, Ovt, ApoA1, Hpx, Ttn and PIT54 were measured using the quantitative proteomic method detailed in Section 4.2.2. Measurement of these samples was undertaken at the same time as those detailed in Chapter 4. For this analysis three replicates per group were randomly selected for measurement owing to cost constraints, as oppose to the six replicates used for the immunoassay measurements.

5.2.3 Apolipoprotein A4 indirect ELISA

5.2.3.1 Western blotting

Western blotting, as described in Chapter 2, Section 2.2.2.2 comparing acute phase and non-acute phase serum samples was undertaken using rabbit anti-APO4 antibody (abcam 113108) at 1 in 10,0000 and 1 in 20,000 dilutions. Jejunal samples, recovered from the birds sampled in the intestinal challenge study were flushed on recovery and frozen immediately. They were individually ground in liquid nitrogen and 0.2g was vortexed in a Tris buffer (20mM, pH7.4). The samples were centrifuged and the supernatant recovered. A 1DE gel with intestinal lysate or serum diluted 1:10 with dH_2O from two challenge samples and two control samples from days 18 and 22 were run. Western blots performed as previously described using was goat α-rabbit IgG HRP (Ab 6721) diluted 1:20,000 as a secondary antibody.

5.2.3.2 Indirect ELISA

Samples were diluted at 1:10,000 or 1 in 20,000 in coating buffer (50mM Sodium hydrogen carbonate, pH 9.4) and 100µl was added in triplicate to a 96 Maxisorp 96 MicroWell™ plate (Nunc International, Rochester, NY). The standard was a previously determined high sample that was double diluted 1:4,000 – 1:64,000, of which 100µl was added in triplicate to the same plate. The plate was incubated overnight at 4˚C. Following removal of the coating buffer, the plate was blocked with 200µl of 5% (w/v) Marvel milk protein diluted in TBS-T (0.05%) over an hour at room temperature with rocking. The plate was washed with TBS-T (0.05%) - 1% (w/v) Marvel milk protein three times, each wash consisting of agitation followed by thorough removal of wash. Following the final wash, the plate was patted dry and rabbit anti-chicken APO4 antibody (abcam 113108),

was diluted 1:500 with TBS- 1% (w/v) Marvel milk protein and 100µl was added to each well. The plate was incubated at room temperature for 1 hour with agitation. The plate was washed three times as previously described and 100µl of goat anti-rabbit IgG-HRP (abcam ab6721) diluted 1:2000 with TBS - 1% (w/v) Marvel milk protein was added to each well. The plate was again incubated at room temperature for 1 hour with agitation. The plate was washed three times again as previously described before 100µl of IMB peroxidase substrate (KPL 50-76-00) was added. Following a 5 minute incubation at room temperature which was halted with the addition of 100µl 1M HCl, the absorbance was measured at 420nm on a FLUOstar absorbance microplate reader and analysed using associated MARS data analysis software (both BMG LABTECH, Ortenburg Germany). A standard curve using 4-parametre fit curve was used to determine Apo-AIV concentrations as arbitrary units (AU) with 100 AU being the value obtained with the standard at a 1:4000 dilution.

5.2.4 Biomarkers for intestinal health

5.2.4.1 Quantitative proteomics

Using the quantitative proteomic methods described in Chapter 4, Section 4.2.2, apo-AIV and villin, proteins previously determined to be of interest as potential biomarkers of intestinal health were targeted in the initial shotgun analysis. Villin however was only identified in the AP pool (pool-2) and no quantotypic peptides were identified. Instead the protein gelsolin was targeted. Gelsolin was present in all three pools and given the functional similarity and presence of four quantotypic peptides, gelsolin was targeted instead. Plasma apo-AIV and gelsolin were measured by quantitative proteomics in the intestinal challenge study and in the three acute phase groups detailed in Chapter 4, HAP, AP and NAP, to determine whether the serum concentration of these intestinally derived proteins change during an APR.

5.2.4.2 Villin as a serum biomarker for intestinal health

A series of western blots, using mouse anti-chicken villin (MCA292 AbD serotec, Oxford, UK) a monoclonal antibody, were undertaken on both plasma and intestinal lysate. Western blots were undertaken as described in Chapter 2 Section 2.2.2.2, except visualisation of immunoreactivity was undertaken using

Pierce ECL to detect HRP activity (#32209 Thermo Scientific, Paisley, UK) according to the manufacturer's instructions. The NCP was trimmed and wrapped in Saran Wrap and exposed to radiographic film for approximately 3 minutes and the resultant radiographic films scanned.

5.2.5 Statistical analysis

GraphPad Prism v.5 and v.6 were used for statistical analysis. All results were analysed using non-parametric tests: Kruskal-Wallis test with Dunn post-test analysis, Mann-Whitney test and Spearman's correlation, following data evaluation using D'Agostino-Pearson omnibus normality test. Non-parametric analysis was selected based on the results together with the small number of replicates in each group.

5.3 Results

5.3.1 Intestinal challenge study

As reported in O'Reilly (2013), there were no mortalities during the study though by day 18 it was evident that the introduction of the challenge litter at day 12 had affected the growth of the challenged pens with uneven growth evident. The mean weight for the challenged birds, when compared to un-challenged controls at day 22 was significantly lower ($P<0.05$), with mean body weights of 374g (± 41g) and 441g (± 34g) for the challenged and control birds respectively. A representative sample of the re-used litter, submitted for microbial analysis on the day of collection identified *Clostridium perfringens*, *Lactobacillus* and *Campylobacter*. The oocyst count from the re-used litter was 27,000 oocyst per gram which corresponding to a lesion score 1 (Janseen Pharmaceutica, 1990). This oocyst count is within the expected range for a poultry unit at 35 days (O'Reilly, 2013).

5.3.2 The effect of intestinal challenge on the plasma concentration of acute phase proteins

5.3.2.1 Immunoassays

Plasma concentrations of SAA and Cp were determined using commercially available immunoassays and Ovt concentrations were determined using RID as detailed in Chapter 3. None of these APPs were found to be significantly different between the challenged and control groups at any time point and the plasma concentrations of these APPs were not significantly affected by time. Figure 5.4 details graphically the results of these APP immunoassays. Using the same samples serum AGP concentrations were also determined in an earlier study, and were not significantly different between treatment groups at each time point but were significantly affected but time, increasing significantly over time in both treatment groups (O'Reilly, 2013).

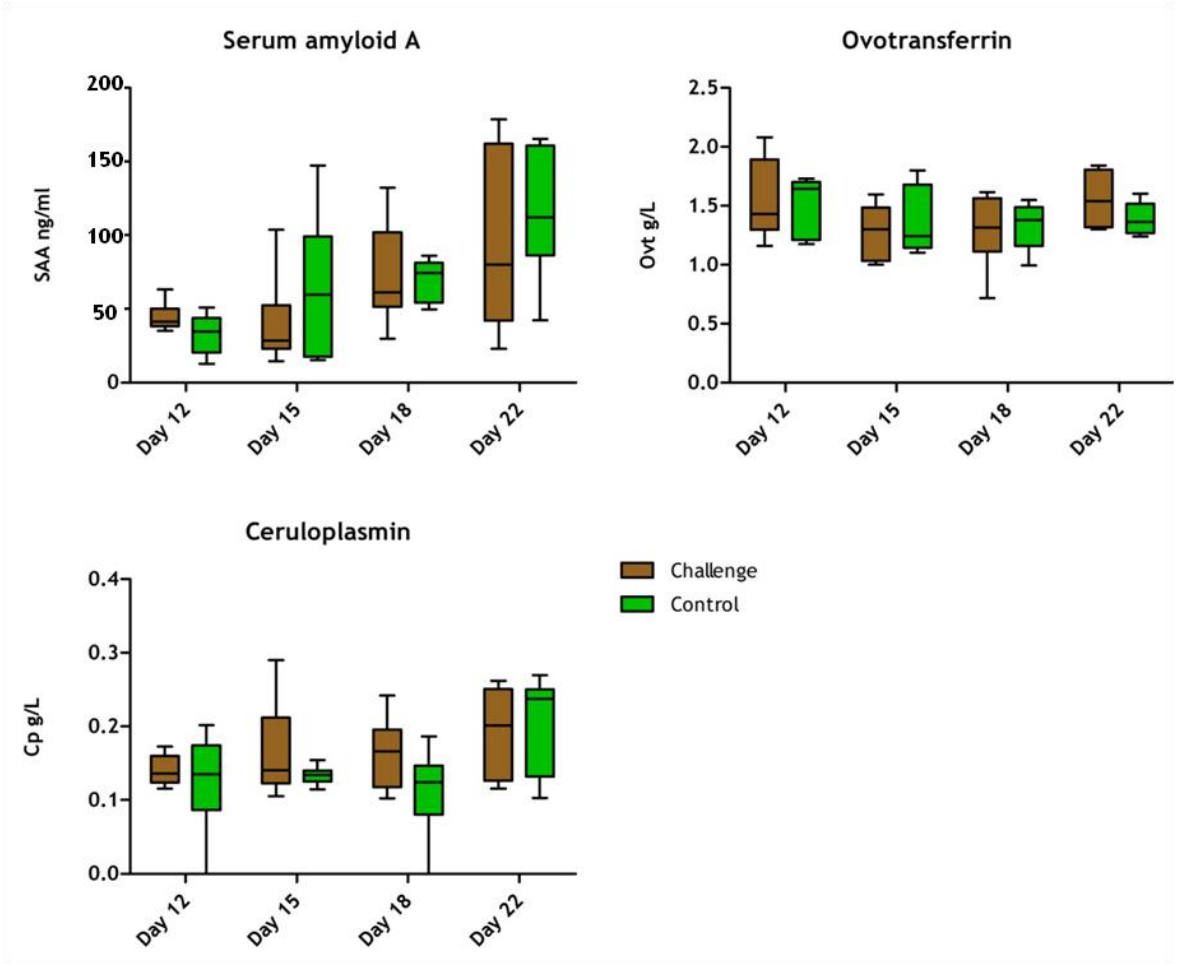


Figure 5.4: Boxplot graphs describing serum concentrations of SAA, Ovt and Cp, determined by immunoassay, over time in challenge (n=6 per time point) and control groups (n=6 per time point). Data was analysed using Kruskal-Wallis with Dunn post test analysis to identify any significant effect of time for both control and challenged groups (analysed separately). At each timepoint a Mann-Whitney test was performed to identify any significant difference between the control and challenged birds at that time point (GraphPad Prism v5). Data presented in median with 25 to 75 percentile range.

5.3.2.2 Quantitative Proteomics

The same methods, used successfully in Chapter 4, Section 4.2.2, were used to quantify the APPs SAA, Ovt, ApoA1, Hpx, Ttn and PIT54 in three replicate samples from the challenge and control samples at each time point. No significant differences between the challenge and control samples was seen for any of the APPs at any time point except for Hpx where peptide-1 in the control group was significantly different over the four time points, increasing from day

12 to day 22. A similar, but non-significant trend was seen in the challenge group and in both challenge and control groups for peptide-2.

5.3.3 Apolipoprotein AIV

5.3.3.1 Western blotting

The western blots worked at both antibody concentrations with specific binding producing a distinct band at 38kDa and no noted background binding. The molecular weight of chicken apo-AIV is reported to be 40.8 kDa. There were no detectable difference between the acute phase and non-acute phase samples at either dilution. Figure 5.5 shows the western blot, at a 1:10,000 dilution it is possible to detect a second slightly heavier band. A negative control was undertaken and no binding was identified.

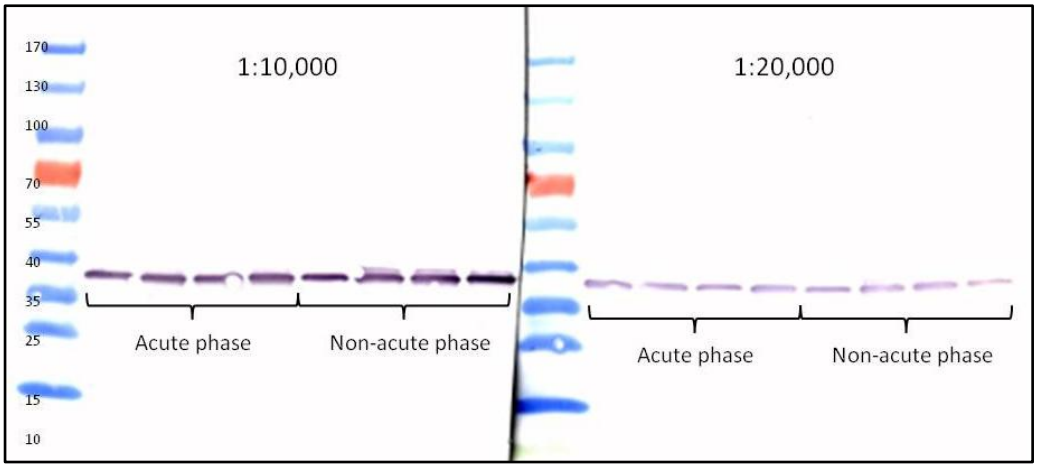


Figure 5.5: Western blot comparing apo-AIV in acute and non-acute phase samples using rabbit anti-chicken apoIV antibody diluted 1:10,000 and 1:20,000. Serum proteins were separated with 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124) prior to western blotting.

Western blotting was also undertaken with intestinal lysate with bands apparent at the 38kDa, the same molecular weight as the serum apo-AIV. The bands however were not as intense (hence image not shown). There were no obvious differences between the size and intensity of the bands between the lysate samples from the two days (18 and 22) and between the challenge and control.

5.3.3.2 Apolipoprotein AIV indirect ELISA

5.3.3.2.1 ELISA validation

To develop and validate an indirect ELISA for the measurement of apo-AIV in serum and plasma, a number of steps were undertaken to determine the optimal serum and antibody concentrations. The optimal antibody concentrations for the rabbit anti-chicken apo-AIV antibody were determined by diluting the antibody 1:100, 1:500, 1:2000 and 1:8000. Figure 5.6 plots the OD of five serum samples, serially diluted fourfold from 1:4000 to 1:256,000, calculated from standard curves produced at four different antibody concentrations. The samples show linearity but plateau at both high and low dilutions. Based on these findings a 1:500 antibody dilution was selected as the most optimal antibody concentration producing the best standard curve. The same high sample double diluted from 1:4,000 to 1:64,000 was used to produce all subsequent standard curves used to calculate apo-AIV concentrations in this study. Arbitrary units (AU) (in the absence of a purified sample of known concentration) were used to record the concentration of each sample. The calculated standard curve is based on 1:4000 equalling 100% through to the 1:64,000 sample equalling 6.3%, using a logarithmic scale along the X axis (Figure 5.7, Table 5.1). The apo-AIV concentration of two samples (previously determined as containing relatively high and low apo-AIV) were diluted 1:10,000 and 1:20,000 and apo-AIV concentrations determined, the results presented in Table 5.2. From this data samples were measured at a 1:10,000 dilution, though in later studies this increased to 01:20,000 owing to higher apo-AIV serum concentrations.

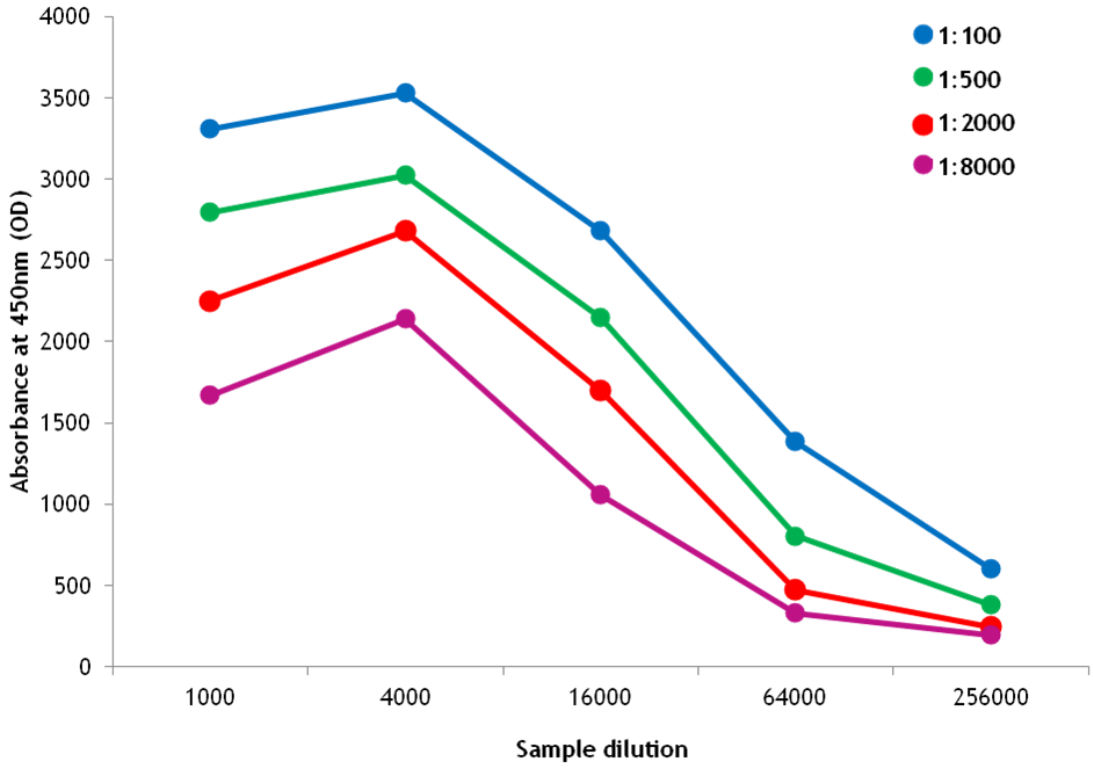


Figure 5.6: Optimisation of antibody concentration for apo-AIV ELISA. Serum was serially diluted from 1:1000 to 1:256000 and the OD recorded for each sample using 1:100, 1:500, 1:2000 and 1:8000 chicken anti-apo AIV antibody.

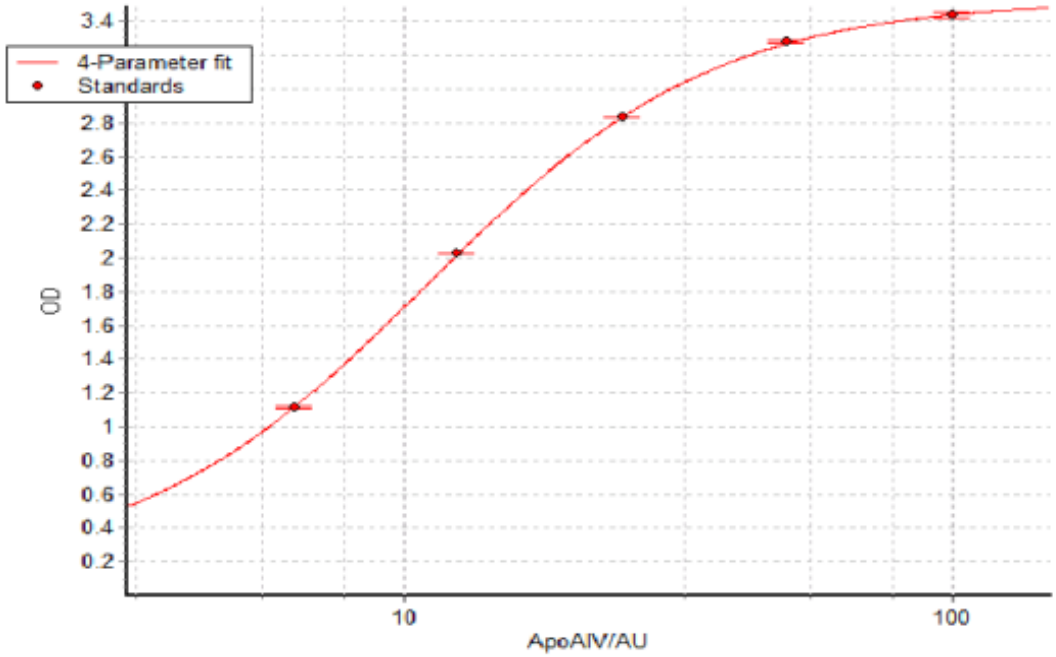


Figure 5.7: Example standard curve using a 1:500 antibody dilution (MARS data analysis, BMG LABTECH). Using the 1:500 antibody (that appeared to give the optimal response as detailed in Figure 5.6), the ELISA was re-ran and the standard curve plotted using a 4-parametre fit, widely recommended for ELISAs

Absorbance		
Formula:	Y = Bottom + (Top-Bottom) / (1+(EC50/x)^Slope)	
	Fit results are based on OD values	
Wavelength:	Top	3.528287
450	Slope	1.646091
	EC50	10.96318
	log(EC50)	1.039936
	Bottom	0.148144
	R	0.999981
	r^2	0.999962

Table 5.1: Standard curve fit results (MARS data analysis, BMG LABTECH).

	1:10,000		1:20,000	
Sample	Results	SD	Results	SD
High	38.56	3.93	35.55	4.74
	38.83		42.90	
	31.89		34.04	
Low	22.83	5.40	21.98	5.04
	19.95		17.75	
	12.38		11.94	

Table 5.2: Apo-AIV results from a high and low sample replicated three times at a 1:10,000 and 1:20,000.

5.3.3.2.2 Inter and intra-assay precision

The inter- and intra-assay coefficient of variants (CVs) were calculated using 107 samples from two preliminary studies and the intestinal challenge study (only high QC) and are contained in Table 5.3. The average inter- and intra-assay CVs are 4.71% and 3.61% respectfully, making this a particularly precise and repeatable ELISA method.

	High QC		Low QC	
Inter-assay CVs	**Plate**	**Mean ApoA4**	**Plate**	**Mean ApoA4**
Preliminary study - 1	Plate 1	51.32	Plate 1	28.20
	Plate 2	53.06	Plate 2	30.71
	Plate 3	49.96	Plate 3	27.99
	mean	51.44	mean	28.97
	sd	1.55	sd	1.51
	CV	**3.02**	**CV**	**5.22**
Preliminary study - 2	Plate 1	42.64	Plate 1	38.43
	Plate 2	48.92	Plate 2	42.85
	Plate 3	44.60	Plate 3	39.91
	mean	45.39	mean	40.40
	sd	3.21	sd	2.25
	CV	**7.08**	**CV**	**5.57**
Intestinal challenge study	Plate 1	43.67		
	Plate 2	42.18		
	Plate 3	44.43		
	mean	43.43		
	sd	1.14		
	CV	**2.63**		
Average Inter-assay CV		**4.71%**		
Intra-assay CV	**plate**	**CV%**		
Preliminary study -1	Plate 1	3.95		
	Plate 2	4.53		
	Plate 3	4.37		
Preliminary study-2	plate 1	3.20		
	plate 2	2.68		
	plate 3	5.02		
Intestinal challenge study	plate 1	3.09		
	plate 2	2.45		
	plate 3	3.21		
Average Intra-assay CV		**3.61%**		

Table 5.3: Inter and Intra-assay CVs of Apo-AIV indirect ELISA. The inter-assay CV was calculated from running 107 samples in duplicate from three seperate studies, across nine plates. The intra-assay CV was calculated using the same studies, measureing all samples in duplicate.

5.3.3.2.3 Limit of detection

The minimum detection limit was taken as the apo-AIV concentration at 3 SD from the mean of blank samples (n=20). This resulted in a limit of detection of 1.56 AU.

5.3.3.2.4 Plasma apolipoprotein AIV in intestinal challenge study

Using the indirect ELISA, the plasma concentrations of apo-AIV was determined in all the samples in the intestinal challenge study. Figure 5.8 shows the concentration of apo-AIV over time in challenged and control samples. A Mann-Whitney non-parametric test was performed between the challenge and control groups at each time point and revealed no significant difference in Apo-AIV plasma concentrations. Kruskal Wallis tests, performed between the four time points for the challenge and control groups revealed however that for the control group there was a significant effect of time (*P*=0.0441), with Dunn post test analysis revealing the significance to lie between day 12 and 15 (P=<0.05). No significance was detected over the four time points for the challenge group.

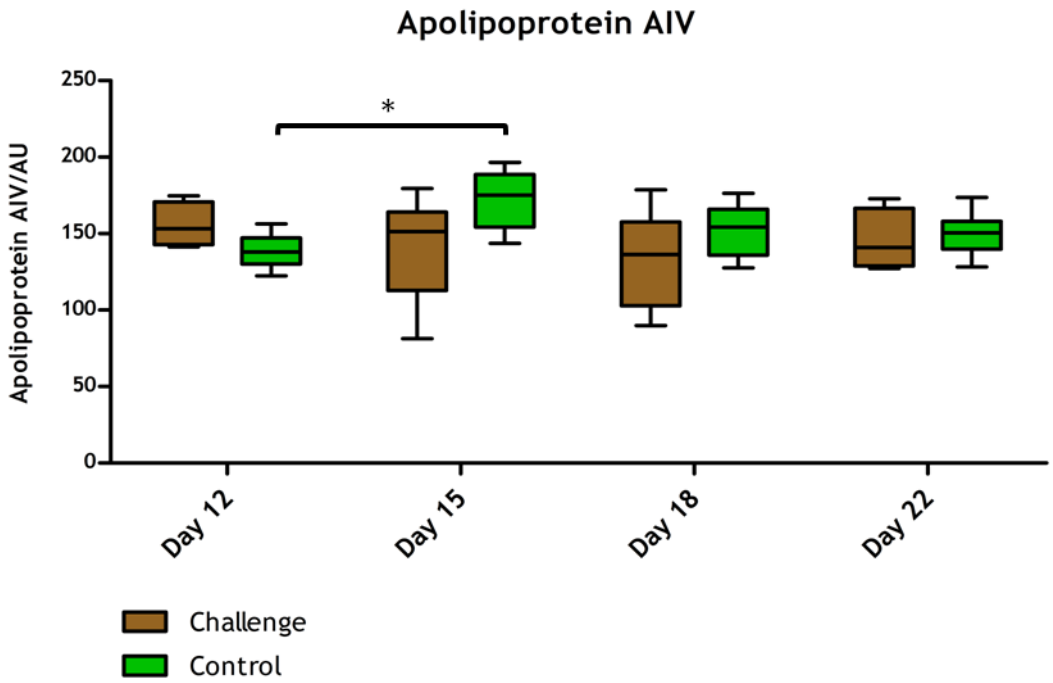


Figure 5.8: Boxplot graphs describing plasma concentration of Apo-AIV, determined using indirect ELISA, in challenge (n=6 per time point) and control groups (n=6 per time point). Data was analysed using Kruskal-Wallis with Dunn post test analysis to identify any significant effect of time for both control and challenged groups (analysed separately). At each timepoint a

Mann-Whitney test was performed to identify any significant difference between the control and challenged birds at that time point (GraphPad Prism v5). Data presented in median with 25 to 75 percentile range.

5.3.4 Targeting biomarkers for intestinal health

5.3.4.1 Quantitative proteomics

The same quantitative methods detailed and presented in Chapter 4 were used to target apo-AIV and gelsolin in the intestinal challenge study, with a view of identifying possible biomarkers of intestinal health. Table 5.4 details the quantotypic peptides targeted. For apo-AIV no significance was noted between challenge and control groups at any of the time points. When examining the challenge and control groups over time however a Kruskal-Wallis test showed a significant difference between the time points (P=<0.05) in the challenge group, Dunn post-test analysis revealing this to lie specifically between days 12 and 18 (P=<0.01) and days 15 and 18 (P=<0.05). The corresponding test for the control group revealed no significance. For gelsolin no significant difference between the challenge and control groups at any time point for any of the four peptides. No significance was identified in the control or challenge groups over the four time points for any of the gelsolin peptides. Figure 5.9 shows the peak area of each peptide for each group for both apo-AIV and gelsolin.

	Peptide 1	Peptide 2	Peptide 3	Peptide 4
Gelsolin O93510	K.NLYGDFFTGDSYLVLNTIR.Q [79, 97]	K.VPVDPATYGQFYGGDSYIILYDYR.H [453, 476]	R.AVELDPAASQLNSNDAFVLK.T [573, 592]	R.GSNSAELSGAQELLK.V [604, 618]
Apo-AIV O93601	R.LVPFATELQAQLVQDSQR.L [78, 95]	K.LAPYADEVHQQIGTNIR.E [111, 127]	R.AQLSPLAQELQEALR.G [263, 277]	

Spearman's correlations		r^2-value	*p*-value
Gelsolin	*Peptide 1 and 2	0.4791	0.0178
	Peptide 1 and 3	0.3122	ns
	Peptide 1 and 4	0.3035	ns
	*Peptide 2 and 3	0.4800	0.0176
	Peptide 2 and 4	0.3017	ns
	***Peptide 3 and 4	0.7391	<0.0001
Apo-AIV	***Peptide 1 and 2	0.8162	<0.0001
	**Peptide 1 and 3	0.5583	0.0056
	Peptide 2 and 3	0.3557	ns
Apo-AIV ELISA	Peptide 1	0.3350	ns
	Peptide 2	0.2935	ns
	**Peptide 3	0.4575	0.00282

Table 5.4: Quantotypic peptides targeted for apo-AIV and gelsolin quantification and results of Spearman's correlations between each peptide and between each apo-AIV peptide and the results of the apo-AIV ELISA. ns = no significance, *<0.05, **<0.01, ***<0.001, ****<0.0001, (GraphPad Prism). For both proteins the quantitypic peptides are listed to the right in columns, underwhich the result of the Spearman correlation, undertaken between the peptides for each protein across all the samples to determine whether the peptide peak areas (intensities) for each peptides, tryptically digested from the target protein correlate with each other. Asterisks to the the left of the peptide correlation indications level of significance, with p-value and r^2-value listed alongside.

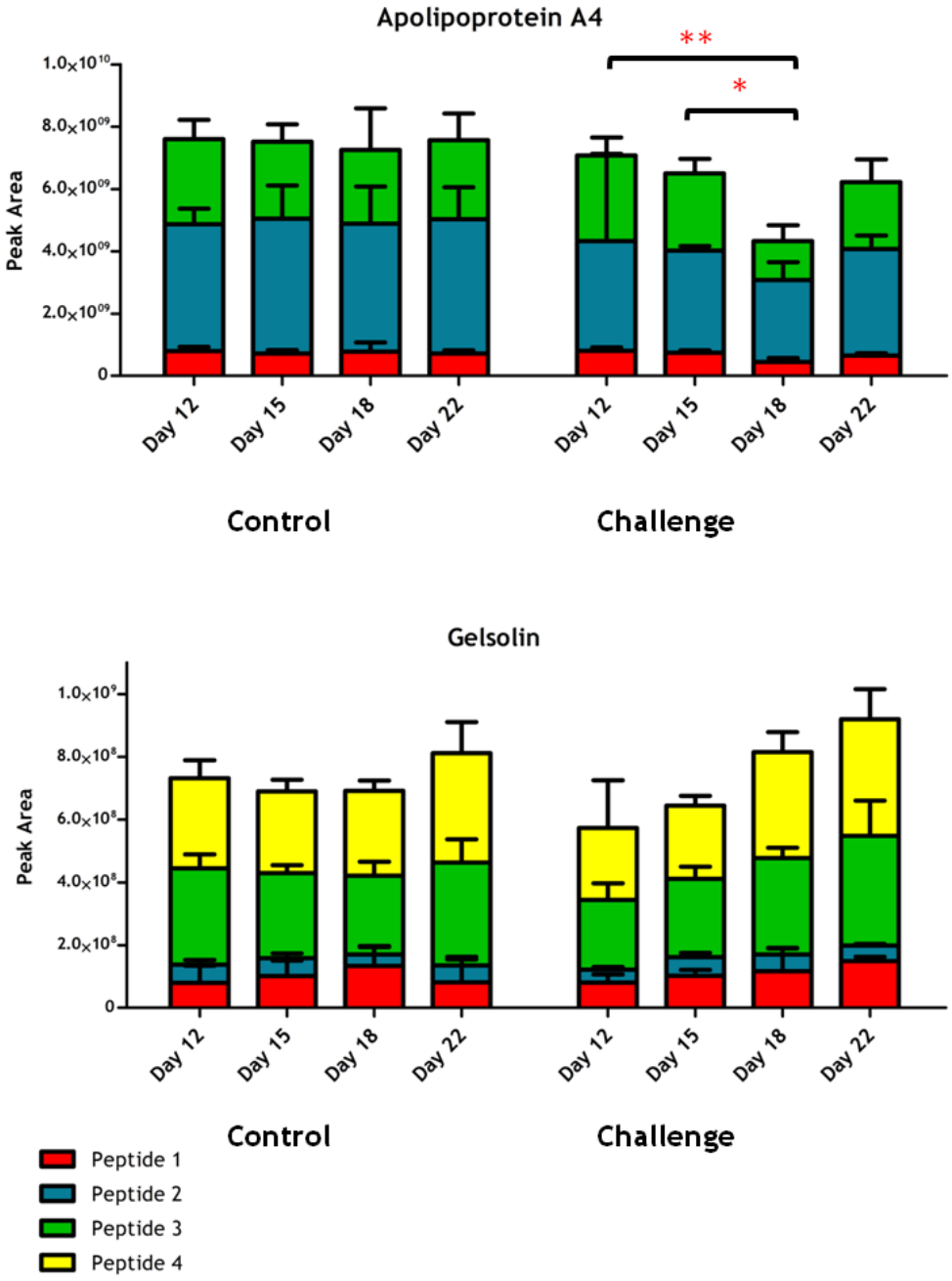


Figure 5.9: Peak area (± SD of the mean) of apo-AIV and gelsolin for challenged (n=6 per time point) and control (n=6 per time point) groups over four time points, divided within bar by peptide. Significance is indicated by asterisk *<0.05, **<0.01. Colour of asterisks corresponds to same coloured peptide as indicated in legend. Data was analysed using Kruskal-Wallis with Dunn post test analysis to identify any significant effect of time for both control and challenged groups (analysed separately). At each timepoint a Mann-Whitney test was performed to identify any significant difference between the control and challenged birds at that time point (GraphPad Prism v5). Data presented in median with 25 to 75 percentile range.

A Spearman's correlation was performed on the results of the apo-AIV ELISA and the peak area value of apo-AIV peptides within each sample. Spearman's

correlations were performed between each peptide to determine whether the quantitative measure of each peptide was relative to the others and as such a good quantotypic peptide. The results of these correlations are contained in Table 5.4 and Figure 5.10. There was no significant correlation between the apo-AIV assay and peptides 1 and 2 from the quantitative proteomics. However for peptide 3 there was a highly significant correlation (p=<0.01). For the peptides themselves there was significance between 1 and 2 and 1 and 3 only.

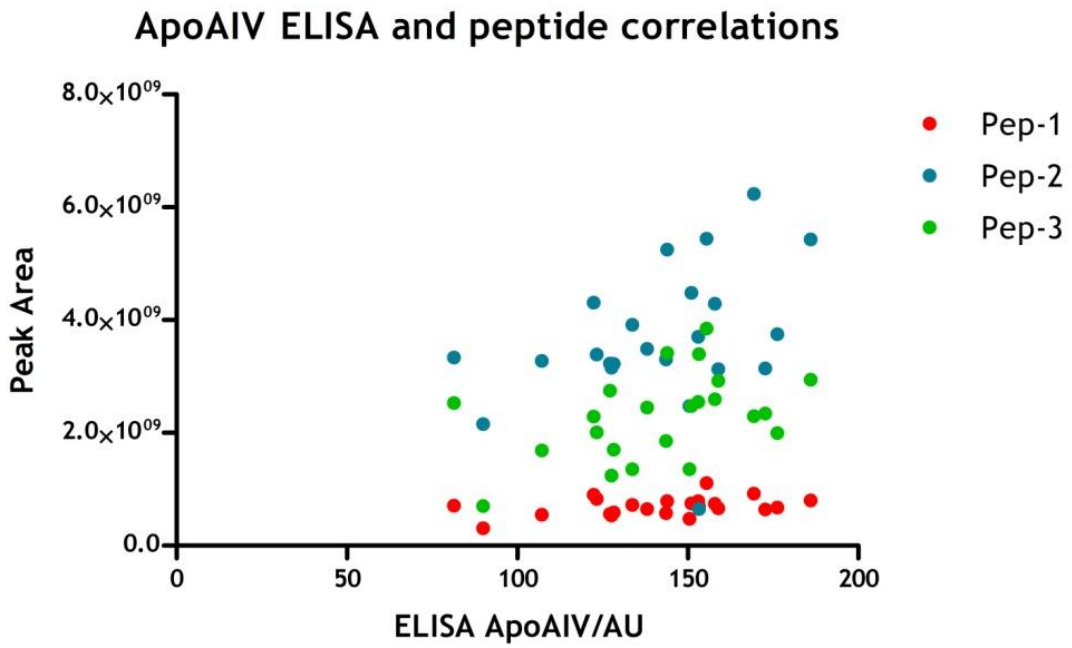


Figure 5.10: X-Y scatterplot of apo-AIV ELISA result (x-axis) and the peak area of each apo-AIV peptide (y-axis) as determined by targeted SIM MS/MS (GraphPad Prism v5).

5.3.4.2 Apolipoprotein AIV and gelsolin during an acute phase response

To determine whether the APR had an effect on plasma concentrations of apo-AIV or gelsolin, these proteins were also measured in the HAP, AP and NAP samples detailed in Chapter 4, using the same quantitative proteomic method. In the same samples apo-AIV was also measured using the ELISA. None of the peptides quantified for apo-AIV or gelsolin were found to be significantly different between the acute phase groups. When measured with the ELISA, the results were the same, no significant differences were observed between the

three acute phase groups. Figure 5.11 details the findings for the ELISA measurement. To investigate further apo-AIV in the context of the APR a Spearman's correlation between the of the immunoassay results of SAA, AGP, Ovt and Cp and the apo-AIV ELISA results from the intestinal challenge study was calculated and the results described in Table 5.5 and Figure 5.12. A significant correlation between apo-AIV and Cp was identified. No other significant correlations between apo-AIv and the other APPs were identified.

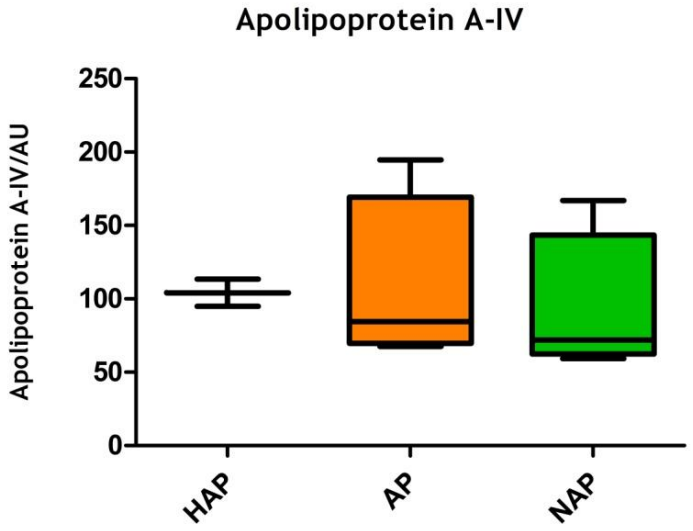


Figure 5.11: Boxplot graph describing apo-AIV concentrations in HAP (n=2), AP (n=4) and NAP (n=4) groups. Data are presented in of median range. Data analysed using Kruskal-Wallis test (GraphPad Prism v5), no significance between the tree groups was detected.

Apolipoprotein -AIV		SAA	AGP	Ovt	Cp
Spearman's correlation	*p*-value	0.6606	0.1283	0.3363	0.0442
	r-value	-0.06808	-0.2356	0.1484	-0.3049

Table 5.5: The results of a Spearman's correlation between apo-AIV and four APPs, measured by immunoassay (GraphPad Prism v5).

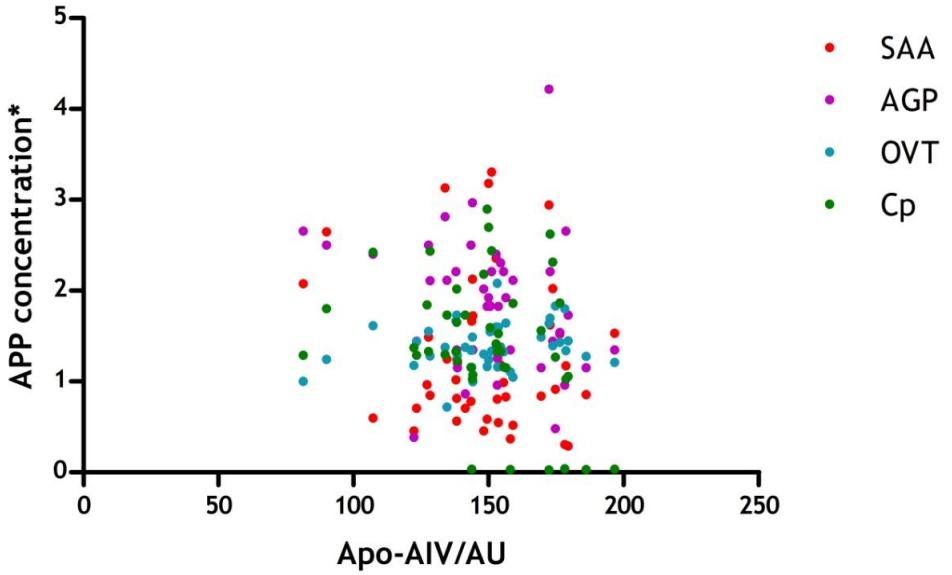


Figure 5.12: X-Y scatterplot of the APPs SAA, AGP, Ovt and Cp (y-axis) and apo-AIV ELISA (x-axis) results for each sample from the intestinal challenge study (GraphPad Prism v5). *The concentrations of these APPs have been adjusted to fit all points clearly on graph: SAA = μg/ml, AGP*=mg/mlx10, Ovt=mg/ml and Cp*=mg/mlx10.

5.3.4.3 Villin as a serum biomarker for intestinal health

Western blotting was carried out on both plasma and intestinal lysate, with a view of demonstrating that the mouse anti-chicken villin antibody was specific and to determine whether it would validate the differential expression of villin in the intestine and also whether villin was detectable and quantifiable in the plasma. Figure 5.13, a western blot of pooled intestinal lysate derived from challenged and control samples over the four time points, shows clearly that this antibody works well with no non-specific binding evident with samples or the negative control. The day 12 control sample did not work well because of difficulties ascertaining protein concentrations in this lysate sample. In plasma, despite multiple attempts with a variety of plasma and serum samples, no villin was identified on western blotting.

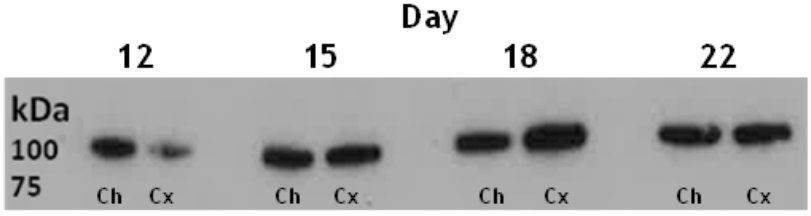


Figure 5.13: Western blot of pooled intestinal lysate from days 12 to 22 for challenged (Ch) and control (Cx) groups with mouse anti-chicken villin antibody.

5.4 Discussion

5.4.1 The effect of an intestinal challenge on acute phase proteins

The results from both the immunoassays and the quantitative proteomics show that the introduction of re-used poultry litter at day 12, was not sufficiently immunogenic to evoke a rise in the APPs SAA, Ovt, Cp, Apo-AI, Ttn and PIT54. For Hpx both peptides appeared to increase over time in both the control and challenged groups, with one of the two peptides measured found to be significantly higher between day 12 and 22 in the control group. An increase over time in both treatment groups was identified with the APP AGP, which increased in all groups over time regardless of treatment group (O'Reilly, 2013). While no studies have sought to determine the normal age related changes in APP concentration in chickens, it is possible that an increase in some APPs during the early growth period is a normal finding in chickens. Most maternally derived antibodies for vaccinated parent flocks are depleted by day 10 (Gharaibeh *et al.*, 2013), which together with the exposure to an environment that is becoming more microbiology complex could cause a normal, protective and non-pathological rise in some APPs. For SAA there is also an upward trend in both treatment groups, which could possibly have become significant if the trial continued. A further consideration in this study is the experimental model employed. Adding re-used poultry litter, while providing immunogenic stimulation sufficient enough to cause decreased and uneven growth is a generalised challenge and was not sufficient enough to stimulate an APR. Other enteric models however could be more stimulatory to the APR. In an experimental model of sub-clinical necrotic enteritis, Cp was found to increase significantly in challenged birds compared to unchallenged controls, increasing significantly from 1 day to 4 days post-infection (Saleem, 2013). As such it is possible that APPs could be suitable biomarkers for some enteric diseases, though the behaviour of APPs in normal, healthy growing chickens needs to be established.

5.4.2 Apolipoprotein AIV

The apo-AIV antibody was determined to be a highly specific antibody producing no background noise and excellent ELISA repeatability, detecting apo-AIV in both the plasma and intestinal lysate. While the apo-AIV bands were faint with the intestinal lysate, moving to an ECL method which uses the chemiluminescence reaction of HRP to detect the secondary antibody would allow this band to be visualised better on scanning. One notable downside of the apo-AIV ELISA was the high antibody concentration required for the ELISA to be optimal, which made it expensive to perform. It is possible that further work to optimise this ELISA to work equally well at with lower antibody concentrations may yield it to be a more financially viable ELISA which can be applied to large numbers of samples. Shotgun proteomic analysis revealed three apo-AIV peptides to be quantotypic and these were targeted not only in the intestinal challenge samples but also in the HAP, AP and NAP samples from Chapter 4, with a view of determining whether apo-AIV is an acute phase responder in chickens. There was no significant difference between the challenged and control groups or over time for both groups for peptides 1 and 2. Peptide 3 however was significantly lower in the challenged group at day 18, than at day 12 and day 15. The apo-AIV ELISA showed no significance between the challenged and control groups at each time point, though for the control group, apo-AIV was significantly higher at day 15 than at day 12. When examining the correlations between the three peptides and the ELISA results, only peptide 3 was significantly correlated with the ELISA results (p=<0.01).

There are a number of possible reasons as to why the results from the ELISA and the quantitative proteomics differed. The most likely explanation is the number of samples used in each of the analysis. The ELISA used all six replicates from both the challenged and the control group, while the quantitative proteomic study used only three because of resource constraints. This difference could affect the results. Furthermore, 25% of apo-AIV in plasma is reported to transfer to HDL, the rest found in the lipoprotein-free fraction of plasma (Vowinkel *et al.*, 2004). It is possible that HDL binding could affect antibody binding, as it is reported to do so in SAA and thus affect the detectable levels of apo-AIV in the ELISA.

Despite the differing results, it is clear that the introduction of the litter had an effect over time on the apo-AIV concentrations of either group or both groups. It is also possible that both results are correct, given that they are not contradictory and a significant correlation, at least between one of the peptides and the ELISA was observed. In the control group the increase observed between days 12 and 15, if a normal finding at this age group, is absent in the challenged group. The presence of a higher apo-AIV is indicative of satiety, the ingestion of lipid having triggered the synthesis and release of apo-AIV. The increase seen in the control birds could be normal, indicative of the birds growing and feeding normally. The absence of a similar increase between the same time points in the challenged group could therefore be indicative of reduced feed intake, digestion or absorption which resulted in reduced plasma apo-AIV, the absence of a significant increase between days 12 and 15, the uneven growth observed at the later stages and the significant difference in pen weights between the two treatment groups at day 22. While not possible to draw too much from the quantification of just one peptide, the significant changes in and the correlation with the ELISA; together with the significant correlation of peptide 3 with both peptides 1 and 2, mean that the results of this peptide warrant discussion. From days 12 to 18 peptide 3 is significantly reduced in the challenge group, which when taken together with the rest of the results is further evidence that plasma apo-AIV was affected by the intestinal challenge.

There is accumulating evidence indicating that lipoprotein metabolism is strongly influenced by inflammation, infection, and sepsis, with both sepsis and LPS specifically affecting plasma lipoprotein levels by modulating lipoprotein production and clearance through their effect on apolipoproteins, lipolytic enzymes, lipid transfer factors, and lipoprotein receptors (Berbée *et al.*,2005). Apolipoprotein AIV, confirmed to have antioxidant and anti-inflammatory activity (Vowinkel *et al.*, 2004), varies in its response between species. In this study no significant differences between the three acute phase groups was detected, either by ELISA or using quantitative proteomics. Correlating the concentrations of apo-AIV with the APPs SAA, Ovt, AGP and Cp across all the samples in the intestinal challenge study revealed Cp and apo-AIV concentrations to be significantly correlated. In obese humans Cp is higher and concentrations of Cp are strongly correlated with serum triglyceride and cholesterol levels

(Cignarelli *et al.*, 1996), which suggests that the correlation seen in this study could be associated with lipid absorption. Measuring apo-AIV using the established model of LPS injection to induce an APR would conclusively determine whether apo-AIV is an acute phase reactive lipoprotein in chickens. The evidence from this study however is suggestive of apo-AIV not being an APP in chickens.

More research has still to be undertaken to fully understand the role of apo-AIV in both mammals and birds. The wide ranging functions performed by apo-AIV during homeostasis and during inflammation may make its use as a biomarker problematic. Transgenic apo-AIV negative mice show no significant abnormalities in lipid absorption or feeding behaviour and exhibit normal food intake, although they do take up more food after a long fast (Simon *et al.*, 2011). Apolipoprotein AIV deficiency does not impact on total fat absorption instead there is an increase in secreted triglyceride-rich apoB-containing lipoproteins. Conversely the transgenic over expression of human apoAIV increases serum VLDL cholesterol and triglycerides in the fed state. The subtle effects of apoAIV deficiency or excess on lipid absorption appear discernible under certain conditions (Simon *et al.*, 2011; Pan *et al.*, 2013).

Despite these concerns, apo-AIV may be a useful biomarker in particular areas of poultry research. Its role as a satiety protein in mammals is established and its measurement during feed trials or behaviour studies may be informative and elude to the physiological role apo-AIV has in birds. It has been shown that serum and intestinal apoAIV protein and mRNA levels exhibit circadian rhythms, being higher in the dark, indicating that apoAIV is involved in the diurnal regulation of lipid absorption and plasma lipid levels (Pan *et al.*, 2013). If apo-AIV in chickens is similarly affected the affect of rearing environment and lighting on feeding and growth could be more complex than previously understood.

Further work is necessary to understand fully the role of apo-AIV in chickens. The circadian expression, its expression during feeding and effect on satiety together with its described role as an anti-inflammatory protein mean that there are many areas in poultry research where the measurement of this protein could be applied. The methods described here, particularly the ELISA, give a validated

mode of measurement by which the function of this protein and use as a biomarker in chickens can be fully determined.

5.4.3 Gelsolin

Villin was only detectable in pool-2 (AP pool) during the initial shotgun analysis and it was not possible to identify any quantotypic peptides and an alternative protein was selected for further assessment. As a member of the same actin binding family as villin, its role as a biomarker in humans and the identification of four quantotypic peptides, gelsolin was targeted as a possible biomarker.

Despite its role in humans as a biomarker, no significance was identified in this study. Four quantotypic peptides from gelsolin were identified and quantified, both in the intestinal challenge study and in the three acute phase groups. Gelsolin was not found to be significantly affected by the introduction of the litter, or the time course of the study. Similarly there was no significant difference in the abundance of gelsolin in the three acute phase groups for any of the four peptides. It appears that in the chicken, gelsolin is not a biomarker of intestinal dysfunction or the APR.

5.4.4 Villin

Plasma villin was targeted for quantitative proteomics due to the changes seen in villin expression in the intestine in birds receiving re-used litter as an immunogenic challenge. Intestinal villin reduced significantly over time when compared to the unchallenged controls appearing to be negatively associated with the challenged group (O'Reilly, 2013). It was the aim of this study to use quantitative proteomics, a sensitive method of detecting peptides and identifying proteins, to measure villin in the plasma in the birds from the same study to correlate the intestinal expression of villin and abundance of villin in the plasma. While villin is synthesised in the intestine, leakage of this protein into the systemic circulation resulting in detectable levels in the plasma and sera is reported (Dudouet *et al.*, 1990). Unfortunately villin was only detected by shotgun analysis in one of the acute phase pools, pool-2 (AP). A mouse anti-chicken villin antibody was used for western blotting of both intestinal lysate and plasma from the intestinal challenge study. While villin was clearly detectable in the intestinal lysate, it was not detectable in the plasma. The results of the western blots indicate that villin is unlikely to be present as a

plasma protein derived from the intestine in chickens that are healthy or receive re-used litter as an immunogenic stimulation. It was detected however in the plasma of the AP pool. Despite none of the six identified peptides being suitably quantotypic, its presence in the serum of chickens mean that it was detectable and is potentially measurable in some intestinal diseases or during an APR. In the intestinal challenge study, there was reduced expression in the intestine and villin may only be detectable (as it is in humans) in the plasma or serum during disease processes that increase the intestinal expression of villin. The AP pool was derived from on farm culled birds with high concentrations of AGP. It is possible that one or more of the samples in this pool originated from a bird with an intestinal disease that increased expression of villin. Using shotgun proteomics to identify villin for quantification would benefit from an enrichment process and given the high presence of villin in the intestine (as determined from the western blotting) the use of intestinal lysate for this purpose could allow many more peptides to be identified.

Until villin is differentially identified in the plasma or serum it cannot be regarded as an intestinal biomarker. Its identification in the serum of an AP pool and its role in maintaining intestinal integrity and function mean that methods to quantify this protein in both intestinal lysate and the plasma of diseased and healthy chickens should be pursued. Its notable differential expression in the jejuna of chickens as a result of the introduction of re-used poultry litter and its identification in serum mean that villin should continue to be a protein of interest in chicken intestinal health.

Chapter 6

Proteomic investigation of gastrocnemius tendon rupture in broiler chickens

6.5 Introduction

Proteomic investigation of diseased and healthy tissue has lead to a major advance in biomarker identification and elucidation of the way in which infectious agents cause diseases in their hosts. Although this advancement is most notable in the area of human health and disease there is increasing momentum in the animal health sciences towards utilising proteomic methods to investigate animal diseases as well as identify biomarkers. Uncovering the proteomic changes between diseased and healthy tissues allows for a deeper understanding of animal diseases and the way different species respond to pathogens. Studying disease at the protein level is challenging and proteins, as mediators of phenotype, require complete understanding if the molecular mechanisms of disease are to be fully understood (Friedman & Lilley, 2008).

Moving forward from plasma proteomics, methods can also be used on whole tissues that have been collected from experimental or clinically affected animals. Tissues are collected, homogenised and the proteins compared. Furthermore, advancement in the way tissues and cells are prepared has allowed for the identification of proteins in tissues or sub-cellular fractions by mass spectrometry methods (Lilley & Friedman, 2004). Sub-cellular fractionation is undertaken to reduce the protein complexity and to pre-select for biologically associated proteins. By separating cell organelles, cytosol and membranes, these different proteomes can be targeted, resulting in a less complex proteomic picture and identification of low abundant proteins (Miller, 2011).

By applying proteomic approaches to the poultry sciences it is possible to closely detail bird responses to disease, dietary interventions and vaccination, and yield useful molecular information. This could assist with disease control by identifying the mechanisms by which pathogens infect birds and have their most deleterious effects.

There are many areas within the poultry sciences that have harnessed the use of proteomics to investigate bird response to diseases, avian pathogens and poultry food products though none to date have examined the tendon proteome of chickens.

6.5.1 The Gastrocnemius tendon

The gastrocnemius muscle (GM) consists of three muscle bellies that lie on the caudal aspect of the tibiotarsal bone. The GM is the main extensor of the intertarsal joint, inserting by a single tendon: the gastrocnemius tendon (GT) onto the hypotarsus, a proximal extremity on the plantar aspect of the tarsometatarsal bone (Figure 6.1) (King & McLelland, 1984).

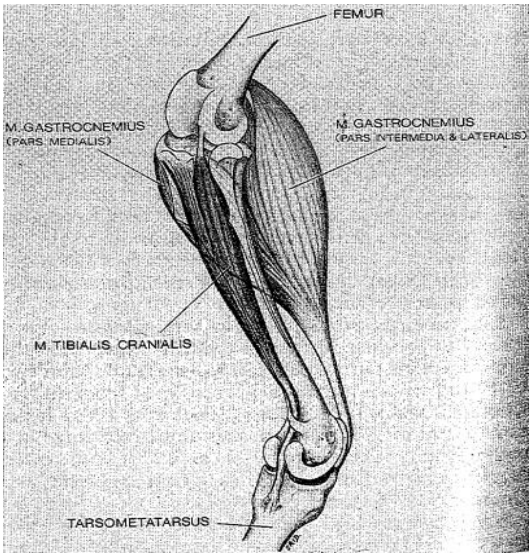


Figure 6.1: Hind limb anatomy of chicken (Duff & Randall, 1986).

6.5.2 Rupture of the gastrocnemius tendon in chickens

Rupture of the GT has long been recognised as a cause of lameness in broiler and broiler breeder chickens with both viruses and bacteria implicated in the aetiology (Duff & Randall, 1986). Avian reoviruses are regarded as the principal aetiological agent, causing tenosynovitis (Jones, *et al.*, 1975, Jones *et al.*, 1981), inflammation of tendons, tendon sheaths, joints and synovial bursae (van Walsum *et al.*, 1981), which causes the GT to rupture. Bacteria and mycoplasmas colonise tendons already damaged by viral tenosynovitis, with staphylococcal infection the most frequent predisposing factor causing secondary GT rupture (Duff & Randall, 1986; Crespo & Shivaprasad, 2011). The aetiological picture of GT rupture however is not completely clear.

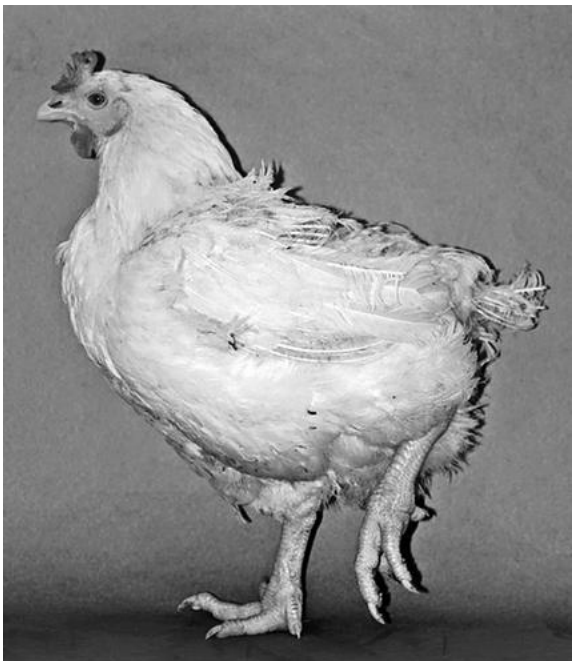


Figure 6.2: Lame broiler chicken with ruptured GT (Crespo & Shivaprasad, 2011).

Gastrocnemius tendon rupture has been found to occur with no evidence of tenosynovitis and studies involving viral isolates tend to induce lesions in tendons other than the GT and are not always re-isolated from lesions, it is therefore suggested that reoviruses do not provoke ruptures alone but do so with the involvement of other predisposing factors (Duff & Randall, 1986; Dinev, 2008). Similarly *Staphylococcus aureus,* considered to be a secondary invader exacerbating a primary reovirus lesion, shows no virulence and tropism for tendon tissue in experimental studies (Kibenge *et al*., 1983).

Gastrocnemius tendon rupture can occur without the presence of infectious agents with non-infectious causes, such as deficiency states, obesity, congenital causes, variations in tensile strength and glucoaminoglycan content hypothesised as causes of GT rupture (Duff & Randall, 1986; Dinev, 2008). Inherited factors resulting from genetic selection for fast growth and increased body weight are reputed to be contributing factors (Duff & Randall, 1986) as GT tendon rupture is seen predominantly in broiler chickens and broiler breeders. Studies into this predisposition are wide ranging and exacting, yielding mixed results. The presence of a avascular area of tendon just above the hock that is the usual site of rupture is a predisposing factor (Duff & Anderson, 1986) and the tensile strength of the GT, an indicator of predisposition to tenosynovitis, has been found to be markedly higher in birds resistant to tenosynovitis (white leghorn

hens) compared to those breeds that are susceptible (broiler breeder cocks). The GT of the resistant birds contain relatively more collagen with collagen fibres showing better alignment and a higher degree of cohesion, this finding particularly observable on the plantar side of the gastrocnemius tendon (van Walsum *et al.*, 1981).

The feeding practices of broiler breeders has also been associated with the incidence of GT rupture with studies finding birds fed *ad libitum* to be affected by GT rupture whereas birds feed restricted diets were not (Riddell, 1983). Conversely Duff & Anderson, (1986) found spontaneous GT rupture to occur in feed restricted birds. Regarding body weight, a case study of an outbreak of GT rupture in broiler breeder hens found no association between body weight and GT rupture, instead associating the GT rupture outbreak with a traumatic aetiology (Crespo & Shivaprasad, 2011). In one experimental study broilers are reported to spend 76% of their time lying down with time spent lying increasing significantly with age (Weeks *et al.*, 2000). The effect of this behaviour and its effect on the GT has been investigated using an immobility model to investigate the effect of disuse on the mechanobiology of broiler GT, revealing that immobility reduces structural strength and toughness of the GT while increasing its material strength and toughness (Foutz *et al.*, 2007).

6.5.3 Difference Gel Electrophoresis (DiGE)

There are a number of issues associated with 2DE such as reproducibility and analytical problems associated with gel to gel variation and a limited dynamic range, all of which severely hamper a quantitative differential display study (Lilley & Friedman, 2004; Friedman & Lilley, 2008). These issues have been circumvented with the development of 2D Difference gel electrophoresis (DiGE) technique, first described by John Minden's lab (Unlu *et al*. 1997).

The DiGE method involves labelling proteins with spectrally distinct fluorescent dyes prior to electrophoretic separation. Cyanine dyes (CyDyes) are chemically modified so that they bind protein and co-migrate with the protein through the gel media (Westermeier, *et al*. 2008) and there are three spectrally distinct CyDyes available: Cy2, Cy3, and Cy5 (DiGE CyDye Fluor Cy™ GE Healthcare). Up to three samples can be bound to each of the three CyDyes, these can then be

electrophoresed on a single gel. The gel is then scanned with a fluorescent imager at different wavelengths and each of the samples on the single gel are then visible separately. Having been ran together under identical condition with proteins migrating under the exact conditions the problems of reproducibility are overcome (Lilley & Friedman, 2004; Unlu & Minden, 2002; Westermeier *et al*. 2008). The sensitive binding of the Cy dyes to the protein together with the use of large 24 cm gels results in a more detailed proteomic image.

6.5.4 Aims of this study

This study aimed to detail the tendon proteome of broiler chickens and to investigate GT rupture in broilers. This GT rupture outbreak appeared unconnected to previously detailed risk factors and no infectious agents had been isolated from affected birds and tendons. The ruptured tendons used in this investigation had previously been examined histologically and a fibrinoid material was found to be associated with the ruptured tendons (R. Bailey *Pers. Com.*). Studies were undertaken to assess whether the tendon material was malleable to proteomic investigations and to optimise the buffers and extraction methods needed to extract soluble protein and separate the proteins according to their molecular weight and isoelectric point. A difference gel electrophoresis (DiGE) method was applied to improve the 2-D separation of the proteins within the tendons samples with a view of detailing the tendon proteome of chickens and establishing whether there are any differences between the proteomes which could elude to the reason as to why the incidence of tendon rupture appears to differ between different groups of birds. It was the hypothesis of this study that proteins, differentially abundant between healthy tendons, ruptured tendons and those from a line of birds with known incidence of tendon rupture, would be identified enabling further elucidation to the aeitiology of GT rupture in broilers.

6.6 Method

6.6.1 Tendon samples

To extract the soluble protein from the tendons, 50g of tendon was cut into small pieces and homogenised using a IKA T25 basic S1 ultra-turrax laboratory homogenizer with 2.5ml of buffer (20mM Tris HCl • 4%CHAPS pH 8.2) for at 17,000 m-1 for 1 minute (Figure 6.3). The tendon was left over night at 4°C to allow the solution to become liquid given the high level of foam formation throughout the homogenate. The homogenate was centrifuged to sink the large tendon pieces and the supernatant recovered. The protein concentration, determined using a Bradford assay as described in Chapter 2, Section 2.2.1.2 found the protein concentrations of the tendons to be low in all samples. To increase the protein concentration the proteins within the supernatant were concentrated using Amicon Ultra centrifugal filters, which have a 3kDa molecular weight cut off (UFC500324, Millipore (U.K.) Limited, Livingston, UK) according to the manufacturer's instructions.

6.6.2 Sample preparation

This study was conducted in association with Aviagen Ltd, a poultry breeding company from where all samples were sourced. The GT of four groups of chickens were studied: ruptured tendons (RT), intact tendon from the opposite leg of the ruptured tendon (IT), healthy tendons from a line of birds known to have incidences of tendon rupture (IR) and control tendons, from a line of birds with no history of tendon rupture (CX). The ruptured tendon samples were from broiler breeder farms. The GTs were recovered from culled birds unable to stand and walk the cause of which was found to be either uni- or bilateral GT rupture. Where the bird was found to be unilaterally affected, the opposite tendon was also sampled and allocated to group 2 (IT). Groups 3 and 4 were taken during routine post mortems of the two lines.

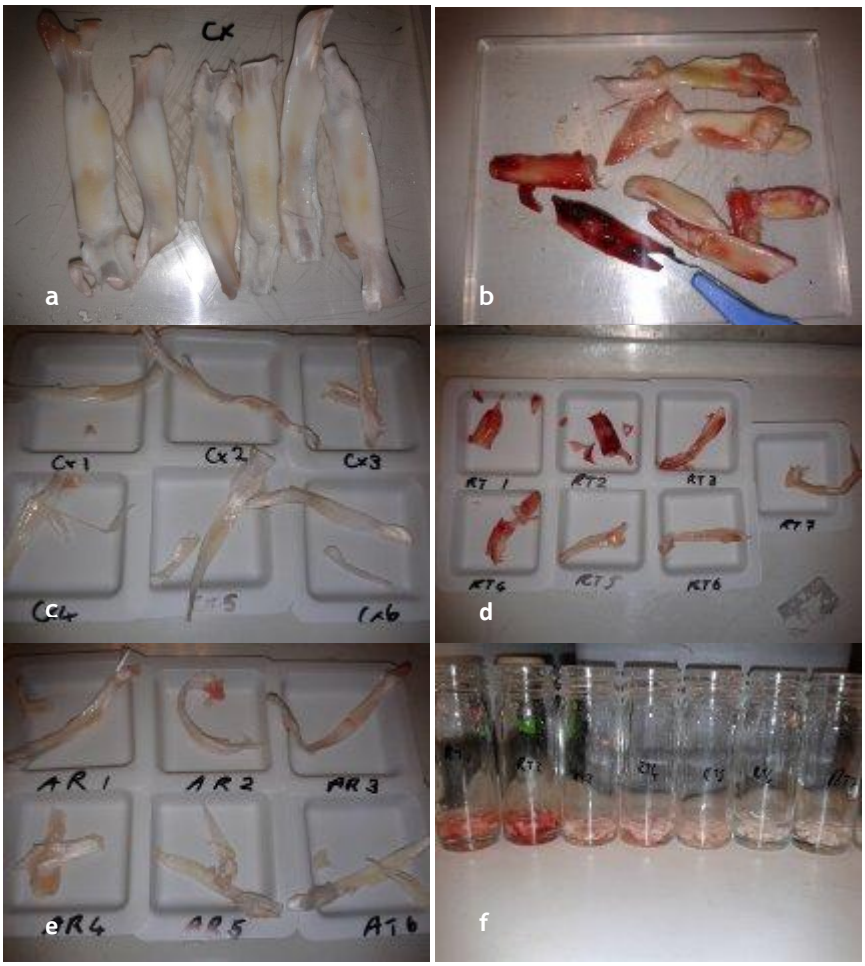


Figure 6.3: Preparing the tendon samples. Control tendons (a) and Ruptured tendons (b) whole before preparation. Control tendons (c) ruptured tendons (d) and known incidence of rupture tendons (e) after weighing. All tendon samples diced and in tendon buffer prior to homogenation (f).

6.6.3 1D and 2D SDS PAGE (1DE/2DE)

To compare the protein profiles of the different tendons, the tendon supernatant, diluted 1:10 with distilled H_2O underwent the same preparation and separated using 1DE and 2DE as described in Chapter 2, Section 2.2.2.1 and where additional steps or deviations to these methods occurred, these are described here. For 2DE gels 180 µg of protein was diluted in rehydration buffer (BioRad 163-2106) and loaded onto 11cm 3-10 pH non-linear IPG strip (BioRad ReadyStripTM) and focused overnight (PROTEIN IEF cell protocol, BioRad). The method for the second dimension is the same as described in Chapter 2, Section 2.2.2.1.

6.6.4 DiGE

6.6.4.1 Sample preparation

For this study the ruptured tendons (RT) (*n*=7), tendons from the line with a known incidence of rupture (IR) (*n*=6) and control tendons (CX) (*n*=6) were prepared as detailed above and pooled to form three pools. To remove organic salts and other impurities that would affect gel quality each of the three pools underwent an acetone precipitation. Pools were diluted 1:6 with DIGE lysis buffer (7M urea, 2M thiourea, 4% CHAPS 25mM Tris) to make a total volume of 200µl. To this 800µl of 100% Acetone at -20°C was added and the samples incubated overnight at -20°C. The pools were briefly centrifuged to sink the hard visible pellet and the supernatant removed. The pellet was re-suspended in 200µl of 80% Acetone at -20°C and the pellet vortexed. This was then centrifuged and the supernatant removed. This wash was repeated twice more. After the final centrifuge the pellet was re-suspended in 200µl lysis buffer. Following the acetone precipitation the protein concentration was measured and adjusted to a concentration of 5mg/ml using a Bradford assay.

6.6.4.2 Preparative gels

Protein spots were picked from the preparative gels. Loaded with a greater concentration of protein, these gels are matched to DiGE gel and spots removed for identification. The preparative gels were produced by mixing 500µg of protein from each three pool with 350µl of rehydration buffer (6M urea, 2M thiourea, 4% CHAPS, 0.002% (w/v) bromophenol blue) into which 3.5mg of dithiothreitol (DTT) and 1.75 µl IPG pH 4 - 7 buffer (GE healthcare biosciences 17-6000-86) was added. These were mixed for 30 minutes, applied to a 24cm 4-7 IPG strip (GE Healthcare Life Sciences 17-6002-32), covered with 1ml of mineral oil and focused to 8000 Volts over 27 hours. Following the focusing the IPG strips was washed in two IEF equilibrium buffer washes for 15 minutes each. The first with 10mg/ml DTT added and the second with 25mg/ml of 2-Iodoacetamide added. For the second dimension the IPG strip was placed onto a 24cm 12.5% SDS-PAGE gel with 1ml of 0.5% agarose with bromophenol blue was added on top of the gel to seal the strip to the gel and to serve as a dye front for monitoring the progress of the gel. The gel was ran in a ETTAN DALT system with

2% SDS running buffer at 1V per gel for 18 hours. The gels were fixed for 1 hour in 40% ethanol / 10%acetic acid, washed twice for 10 minutes in dH2O and stained in colloidal Coomassie for 48 hours.

6.6.4.3 DiGE Gels

The DiGE experimental protocol for the pools and gels are detailed in Figure 6.4. For each of the three tendon pools 50µg of tendon lysate was labelled to both Cy3 and Cy5 DiGE Fluors by mixing 50µg of tendon lysate with 1µl of Cy dye, these were incubated in the dark on ice for 30 minutes. To this 1µl of 10mM lysine was added to stop the Cy's reaction with the protein. For each gel a Cy3 bound sample and a Cy5 bound sample were mixed together, as detailed in Figure 6.4, with rehydration buffer (6M urea, 2M thiourea, 4% CHAPS, 0.002% (w/v) bromophenol blue) into which 3.5mg of dithiothreitol (DTT) and 1.75 µl IPG pH 4 - 7 buffer (GE healthcare biosciences 17-6000-86) was added. These were mixed for 30 minutes, applied to a 24cm 4-7 IPG strip (GE Healthcare Life Sciences 17-6002-32), and focused, washed and ran in the second dimension as described for the preparative gels. Gels were scanned on a Typhoon 9400 laser scanner.

6.6.5 Identification of proteins using mass spectrometry

All spots that were matched from the prep gel to the DiGE gels were picked for protein identification with a view of detailing the tendon proteome of broiler chickens. Spots were removed from the preparative gel and underwent in gel digestion as described in Chapter 2, section 2.2.2.3.1. Similarly proteins were identified using nanoflow HPLC electrospray tandem mass spectrometry (nLC-ESI-MS/MS), in Glasgow Polyomics, in collaboration with Dr R Burchmore. Details of which are described in Chapter 2, section 2.2.2.3.2.

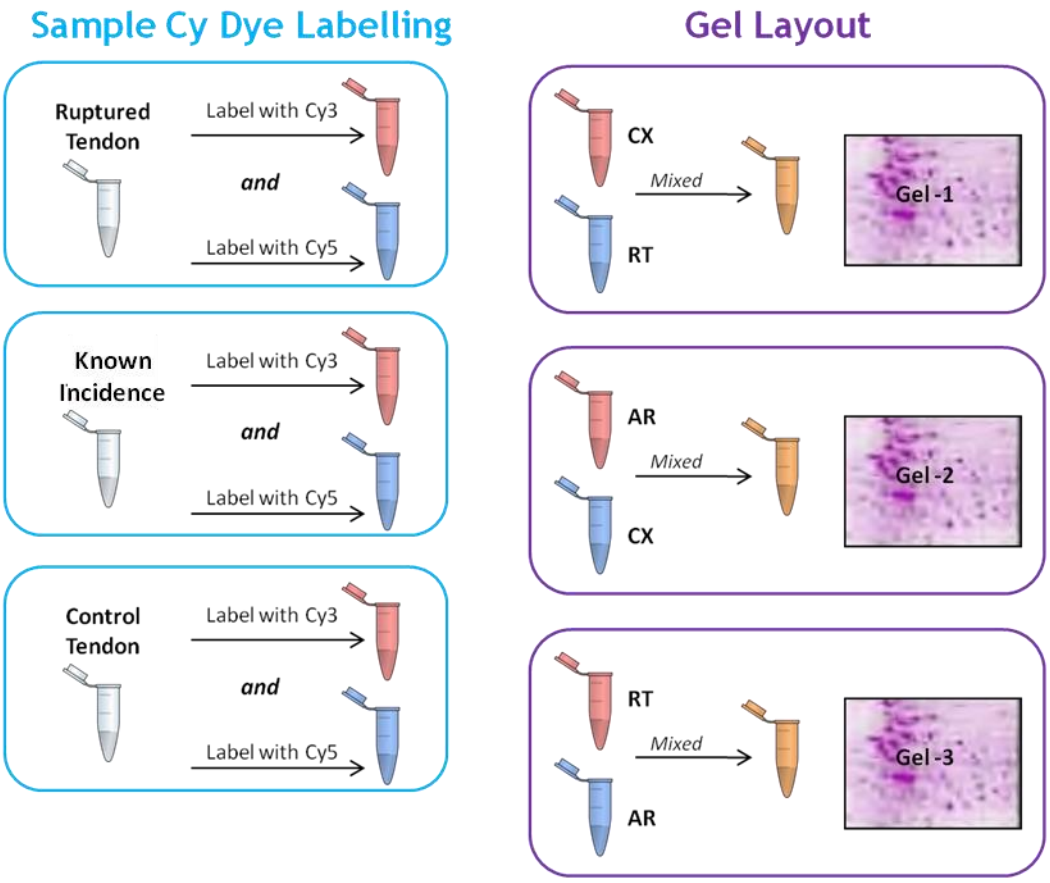


Figure 6.4: Sample labelling and gel layouts. Cy Dye labelling of each of the samples and gel layout of each of the three gels containing two samples with opposite Cy binding.

6.3 Results

6.3.1 1D SDS-PAGE (1DE)

The 1D separation of the tendon lysate was successful, yielding clear bands showing good separation of the proteins by molecular weight. There appeared to be a high degree of homology within each of the groups (Figure 6.5).

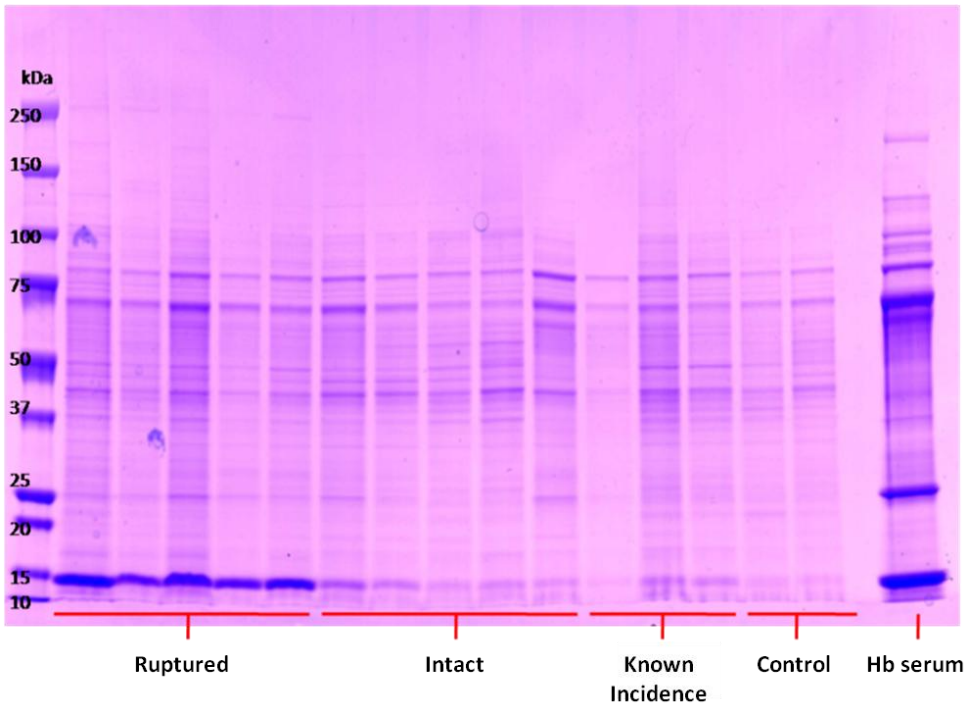


Figure 6.5: Initial IDE gel of four tendon groups. This gel also includes haemolysed serum to confirm the suspicion that the large bands at 15 kDa in the ruptured tendons is indeed haemoglobin. Separation was achieved using a 4-12% CriterionTM Bis-Tris precast polyacrylamide gel (BioRad#345-0124). Protein is stained with Coomassie blue G dye (Sigma Aldrich).

There were also differences between the groups, the most notable of which was intense staining band at 15 kDa for all the ruptured tendons. This was thought to be haemoglobin, from the haemorrhage and inflammation associated with the rupture of the GT. The RT showed gross haemorrhage (Figure 6.3) and this finding on the 1D gel was likely to be haemoglobin, the small molecular weight oxygen transport molecule found with red blood cells. To confirm this haemolysed serum was also ran on a 1D gel and a highly intense band was seen at the same molecular weight in the haemolysed serum, making it highly likely

that this band in the RT is haemoglobin. Gel to gel repeatability was poor for the larger molecular weight proteins within the samples and further gels (not shown) indicated that repeatability to be satisfactory if limited freeze thaw cycles were maintained and the supernatant was well mixed.

6.3.2 2D SDS-PAGE (2DE)

Using 2D SDS-PAGE to separate the proteins within the supernatant over both molecular weight and isoelectric dimensions was achieved. However, the resultant gels were less than optimal, with the gel shown in Figure 6.6 being a more successful example.

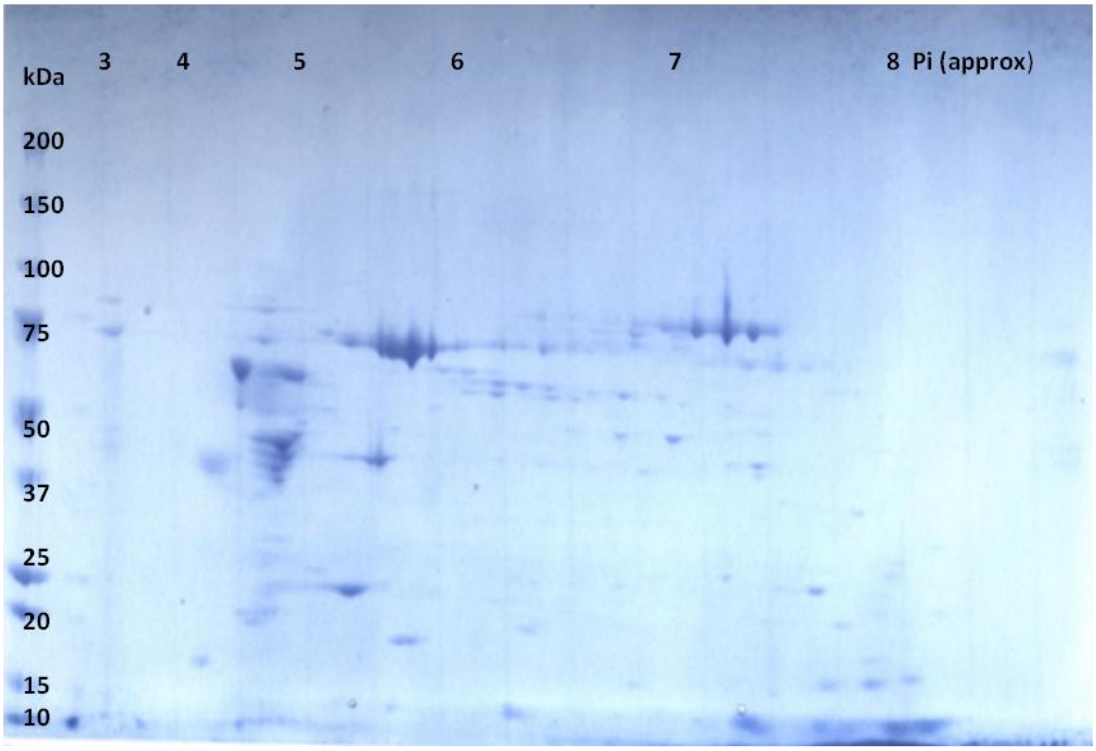


Figure 6.6: 2DE gel of the tendon lysate. Separation was achieved over pi 3-10 and molecular weights of 10-200kDa. An IPG+1 TGX precast midi 4-12% acrylamide gel (BioRad#567-1081). Protein is stained with Coomassie blue G dye (Sigma Aldrich).

Despite repeated attempts and changes designed to optimise the protocol, the repeatability was less than satisfactory. Gels frequently had poor isoelectric focusing resulting in streaking and the control group protein spots were extremely faint. Despite attempts to increase the protein concentration in these

samples stained spots appeared ill defined and flat. To overcome these difficulties, it was apparent that other 2D proteomic methodologies needed to be utilised.

6.3.3 DiGE gels

All the DiGE gels worked well, giving clear, well defined protein separation in both dimensions. Each of the samples, ran in duplicate with a different Cy dyes, exhibited good repeatability. The prep gels, were not as clear and showed some of the problems experienced with the previous 2D method. Figure 6.7 shows each of the DiGE images for each pool with both Cy3 and Cy5 dyes. Gel 1 (Figure 6.7b and f) when scanned was much lighter resulting in less protein detail for both Cy dyes. The preparative gel for CX is shown in Figure 6.8 and this illustrates the less than optimal separation on 2DE gels with higher protein concentrations and Coomassie staining. This resulted in a limited number of spots matched to the DiGE spot maps and therefore identified. A total of 31 spots were excised and identified across the 3 preparative gels. Table 6.1 details the mascot results for the 31 spots excised.

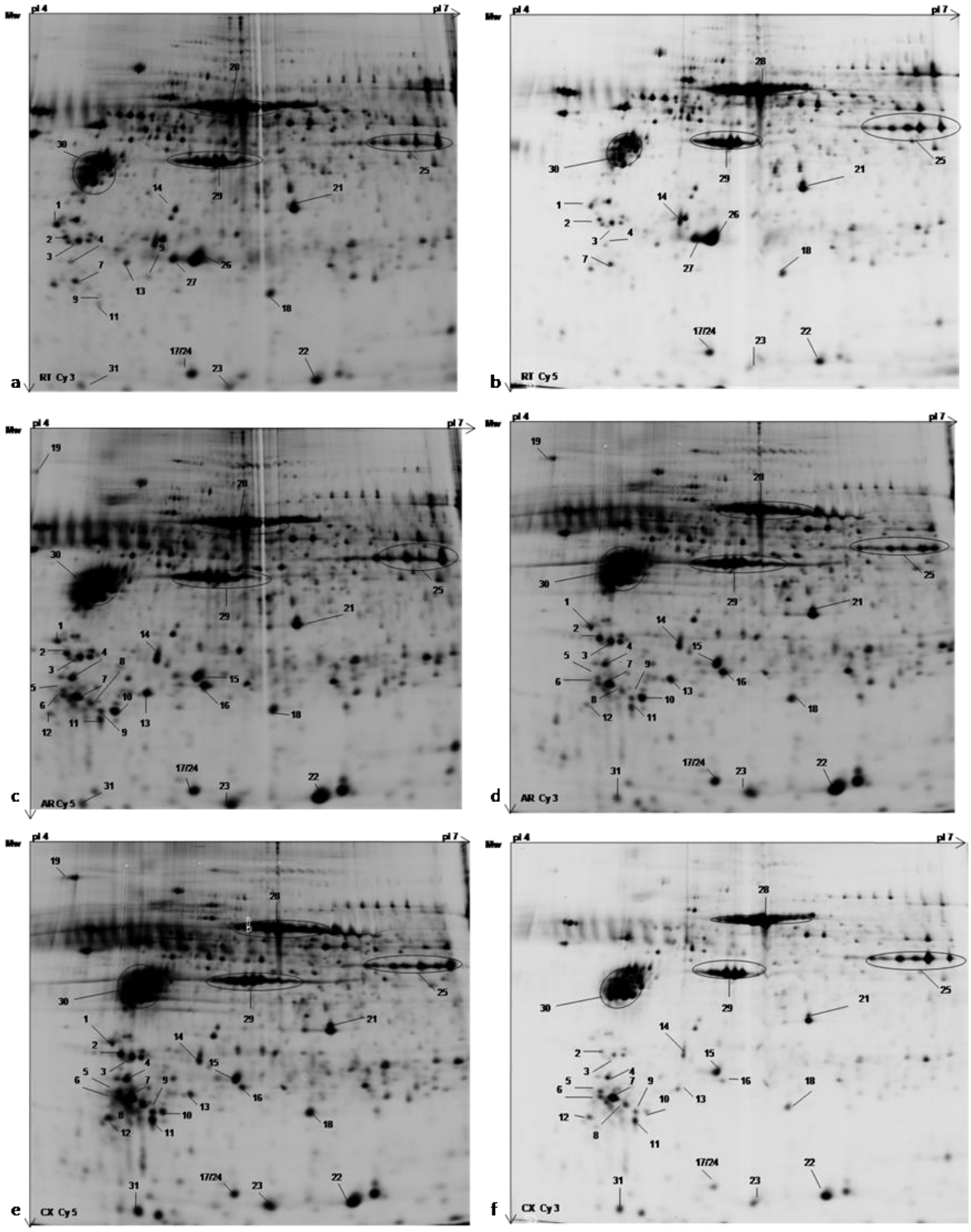


Figure 6.7: DiGE gels in Cy3 and Cy5 for each of the three groups RT (a, b) IR (c, d) CX (e, f). Separation was achieved by binding tendon lysate to Cy3 and Cy5 dyes. Proteins were separated over a pi range of 4-7 using 24cm IPG strip (GE Healthcare 17-6002-32) following reduction and alkylation steps. Following isoelectric focusing the proteins were separated in the second dimension on a 12.5% SDS-PAGE gel.

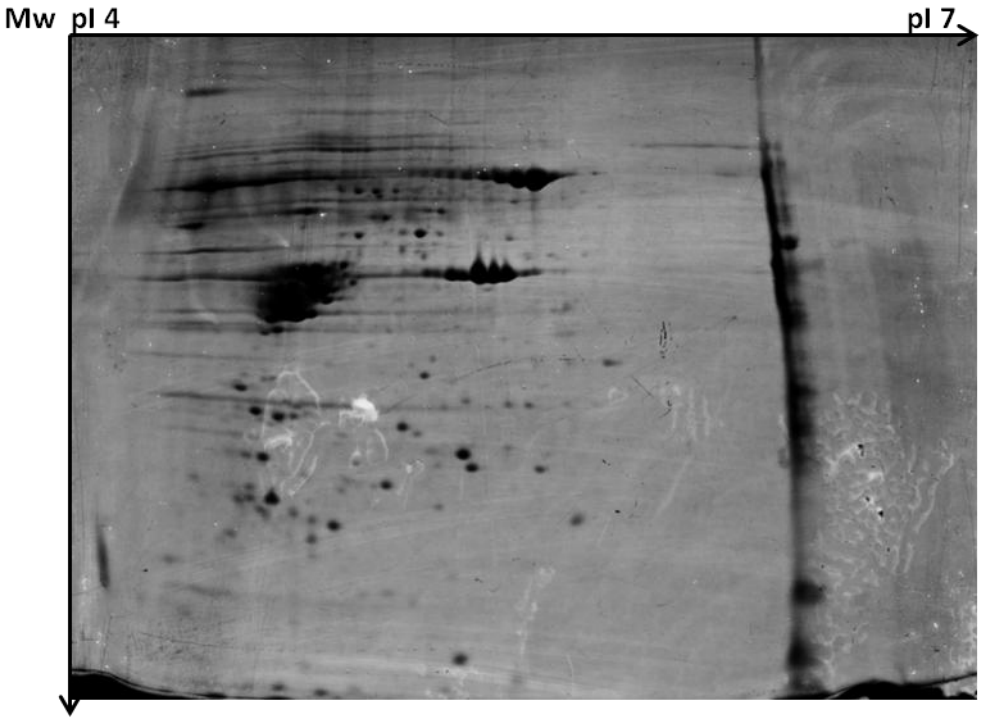


Figure 6.8: Preparative gel for CX. For separation 500µg of tendon was focused over a pi range of 4-7 using 24cm IPG strip (GE Healthcare 17-6002-32) following reduction and alkylation. The proteins were then separated in the second dimension on a 12.5% SDS-PAGE gel. Following spot matching to the DiGE gels, spots were excised from the preparative gels, manually trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates.

ID	Protein ID (*Gallus gallus*)	NCBI Accession	Mr (da)	PI	Peptide matches	Coverage %	Mascot score
1	14-3-3 protein episilon	GI|55741616	29326	4.63	62 (27)	64	714
	14-3-3 protein beta/alpha	GI| 57529350	28004	4.76	9 (3)	10	76
2	14-3-3 protein theta	GI|55741594	28050	4.68	61 (32)	54	627
	14-3-3 protein beta/alpha	GI|7529350	28004	4.76	9 (6)	35	204
	14-3-3 protein zeta	GI|71897035	27929	4.73	30 (11)	33	188
	14-3-3 protein epsilon	GI|55741616	29326	4.63	12 (5)	17	126
	14-3-3 protein eta	GI|56119054	28495	4.18	14 (6)	21	118
3	14-3-3 protein zeta	GI|71897035	27929	4.73	50 (28)	57	633
	14-3-3 protein beta/alpha	GI|7529350	28004	4.76	33 (14)	41	308
	14-3-3 protein theta	GI|55741594	28050	4.68	28 (13)	39	269
	14-3-3 protein eta	GI|56119054	28495	4.81	18 (6)	23	160
	14-3-3 protein epsilon	GI|55741616	29326	4.63	12 (4)	14	79
4	Vimentin	GI|114326309	53167	5.09	73 (35)	38	500
	Desmin	GI|118453	53304	5.45	5 (2)	6	114
5	Vimentin	GI|114326309	53167	5.09	32 (19)	20	427
	Desmin	GI|118453	53304	5.45	7 (2)	4	88
6	Vimentin	GI|114326309	53167	5.09	31 (22)	29	534
7	Vimentin	GI|114326309	53167	5.09	60 (44)	31	852
	Desmin	GI|118453	51689	5.3	11 (5)	7	120

8	Vimentin	GI114326309	53167	5.09	6 (4)	8	110
9	Vimentin	GI114326309	53167	5.09	33(24)	26	529
	tubulin beta-2 chain	GI|363730444	50377	4.78	2(1)	6	95
	Desmin	GI|118453	51689	5.3	4 (2)	7	70
10	Full=Myosin regulatory light chain 2B, cardiac muscle isoform	GI|143811419	18834	4.85	23 (15)	66	339
11	Vimentin	GI|114326309	53167	5.09	25 (17)	24	482
12	Vimentin	GI|114326309	53167	5.09	24 (16)	22	322
13	myosin light chain 1, cardiac muscle	GI|45384044	22096	5.22	38(24)	60	623
	myosin a1 light chain (partial)	GI|212347	19525	4.72	7 (3)	24	129
	myosin alkali light chain	GI|212395	17200	4.63	8 (3)	16	129
	ras-related protein R-Ras2	GI|57530014	23508	5.74	3 (2)	18	111
	apolipoprotein A-I	GI|211159	30673	5.58	5(3)	21	102
	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	GI|118090950	24379	5.84	4 (2)	24	71
14	rho GDP-dissociation inhibitor 1	GI|124249432	23317	5.22	26(8)	58	251
	THY1	GI|30316401	18391	7.6	3 (2)	24	60
	immunoglobulin lambda chain variable region [Gallus gallus]	GI|1536804	10883	4.72	2(1)	23	50
15	apolipoprotein A-I	GI|211159	30673	5.58	25 (16)	70	1105
	Ig light chain precursor V-J region - chicken	GI|104728	22769	5.36	9(3)	24	90
	PREDICTED: gamma-interferon-inducible lysosomal thiol reductase	GI|363743852	28395	5.56	2(2)	12	80
16	myosin light chain 1, cardiac muscle	GI|45384044	22096	5.22	57 (32)	76	1085
	heat shock protein beta-1	GI|45384222	21715	5.77	7(4)	38	120
	apolipoprotein A-I	GI|211159	30673	5.58	7(3)	26	119
	calpain small subunit	GI|2506056	24014	5.05	3(3)	20	109
17	Chain A, Transthyretin	GI|1633502	14209	5.10	30 (19)	76	405
	15 kDa selenoprotein precursor	GI|143771685	18272	5.28	1(1)	10	110
	Chain B, R-State Form Of Chicken Hemoglobin D	GI|4699641	16496	8.85	7(4)	43	101
18	Chain A, The Siderocalin Ex-Fabp Functions Through Dual Ligand Specificities (fatty acid binding protein)	GI|365813051	18276	5.94	24(16)	53	527
	marker protein(fatty acid binding protein)	GI|211503	20204	5.37	21(13)	41	422
19	PREDICTED: cartilage oligomeric matrix protein	GI|118103279	86065	4.27	79(34)	47	874
	PREDICTED: prolargin	GI|118102574	43288	9.11	10(4)	23	137
	decorin precursor	GI|71896943	40061	8.71	5(1)	17	88
20	apolipoprotein A-I	GI|211159	30673	5.58	93(47)	71	1063
	Ig light chain precursor V-J region - chicken	GI|104728	22769	5.36	6(2)	24	78
	PREDICTED: cartilage oligomeric matrix protein	GI|118103279	86065	4.27	3(2)	4	64
21	Chain A, Crystal Structures Of Chicken Annexin V In Complex With Ca2+ (Annexin A5)	GI|62738641	36159	5.61	61(43)	60	856
	F-actin capping protein subunit beta 2	GI|291084676	30820	5.69	7(4)	33	121
22	fatty acid-binding protein, adipocyte	GI|45383556	14999	6.34	41(21)	81	651
	adipocyte-type fatty acid binding protein	GI|33668025	5759	9.37	10(6)	78	151
	Chain B, R-State Form Of Chicken Hemoglobin D	GI|4699641	16496	8.85	3(2)	24	84
23	fatty acid-binding protein, adipocyte	GI|45383556	14999	6.34	30(14)	81	509

	Chain B, R-State Form Of Chicken Hemoglobin D	GI|4699641	16496	8.85	11(8)	49	231
	adipocyte-type fatty acid binding protein	GI|33668025	5759	9.37	8(4)		220
	epsilon globin	GI|71895591	16469	9.2	7(4)	34	157
24	Chain A, Transthyretin	GI|1633502	14209	5.10	14(8)	70	226
	Chain B, R-State Form Of Chicken Hemoglobin D	GI|4699641	16496	8.85	6(5)	50	112
25	alpha-enolase	GI|46048768	47617	6.17	147(75)	70	1943
	hypothetical protein RCJMB04_24e12	GI|53135040	23833	6.97	71(42)	63	1046
	beta-enolase	GI|46048765	47566	7.28	44(21)	23	638
	pre-fibrinogen alpha subunit	GI|732995	56766	6.82	15(5)	35	206
	PREDICTED: cytochrome b-c1 complex subunit 1, mitochondrial	GI|50754375	53410	6.58	10(5)	25	169
	elongation factor 1-alpha 1	GI|54020687	50449	9.10	19(7)	30	167
	ecName: Full=Fibrinogen beta chain	GI|267844833	53272	7.18	21(6)	47	156
	hemoglobin subunit alpha-A	GI|2829707	15533	8.54	12(6)	65	136
	6-phosphogluconate dehydrogenase, decarboxylating	GI|57529439	53771	6.51	6(3)	16	129
26	apolipoprotein A-I	GI|211159	30673	5.58	221(82)	81	1517
	alpha-enolase	GI|46048768	47617	6.17	5(2)	10	106
	Ig light chain precursor V-J region - chicken	GI|104728	22769	5.36	12(4)	24	71
	Ig light chain	GI|212060	23868	5.34	12(4)	22	71
	PREDICTED: gamma-interferon-inducible lysosomal thiol reductase	GI|363743852	28395	5.56	2(1)	12	49
27	apolipoprotein A-I	GI|211159	30673	5.58	192(74)	74	1630
	cathepsin B precursor	GI|46195455	38475	5.74	8(7)	25	248
	Ig light chain precursor V-J region - chicken	GI|104728	22769	5.36	11(4)	24	110
	Ig light chain	GI|212060	23868	5.34	10(4)	22	110
	alpha-enolase	GI|46048768	47617	6.17	2(2)	6	86
	immunoglobulin lambda chain variable region	GI|1536804	10883	4.72	4(2)	38	70
28	serum albumin precursor	GI|45383974	71868	5.51	244(72)	65	1557
	heat shock cognate 71 kDa protein	GI|45384370	71011	5.47	11(55)	22	128
	heat shock protein 70	GI|30962014	70098	5.56	10(2)	15	77
29	actin, cytoplasmic type 5	GI|56119084	42151	5.3	128(43)	76	915
	actin, cytoplasmic 1	GI|45382927	42052	5.29	122 (43)	72	915
	actin, alpha cardiac muscle 1	GI|118405200	42334	5.23	93(4)	46	813
	actin, alpha skeletal muscle	GI|71894831	42366	5.23	92(38)	46	784
	actin, aortic smooth muscle	GI|71895043	42367	5.23	80(36)	46	720
	PREDICTED: actin, alpha skeletal muscle B isoform 2	GI|50803534	42386	5.16	72(32)	38	650
	serum albumin precursor	GI|45383974	71868	5.51	20(6)	37	176
	phosphoglycerate kinase	GI|45384486	45087	8.31	6(4)	18	127
	beta-actin	GI|348167222	16060	5.04	8(3)	26	90
30	Vimentin	GI|114326309	53167	5.09	212(85)	66	1615
	creatine kinase M-type	GI|45382875	43529	6.5	13(7)	25	241
	phosphoglycerate kinase	GI|45384486	45087	8.31	9(5)	23	170
	beta-actin	GI|63018	42080	5.29	17(6)	34	162
	Chain B, Crystal Structure Of The Chicken Actin Trimer Complexed With Human Gelsolin Segment 1	GI|28373640	42340	5.17	13(4)	24	125
	PREDICTED: actin, alpha skeletal muscle B isoform 2	GI|50803534	42386	5.16	11(3)	26	113
31	Vimentin	GI|114326309	53167	5.09	33(26)	38	753
	hemoglobin subunit alpha-A	GI|52138655	15533	8.54	11(7)	57	208

alpha-globin-A	GI|211114	15492	9.1	11(5)	57	142
apolipoprotein A-I	GI|211159	30673	5.58	5(3)	23	113

Table 6.1: Proteins identified in DiGE gels and picked from preparative gels (Figures 6.7 and 6.8). Spots were excised from the gel trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates. Both NCBI and where Uniprot identifications are listed (immunoglobulins are not listed in Uniprot). The protein score is the sum of the highest ions score for each distinct sequence. The first number under matches is the count of MS/MS spectra that have been matched to peptides from this protein. The matches shown in the parenthesis indicating unique matches (scores above the significance threshold). The sequences column is the count of matches to distinct peptide sequences, the number in parenthesis is the count of sequences with significant matches (Matrixscience.com). The coverage percentage is proportion of the protein's entire sequence identified.

Figure 6.9 details the protein results on a typical spot map to give a clearer picture of where the identified proteins are on the gel spot map and to detail the chicken GT proteome. Figure 6.10 is the same image with the spot numbers shown.

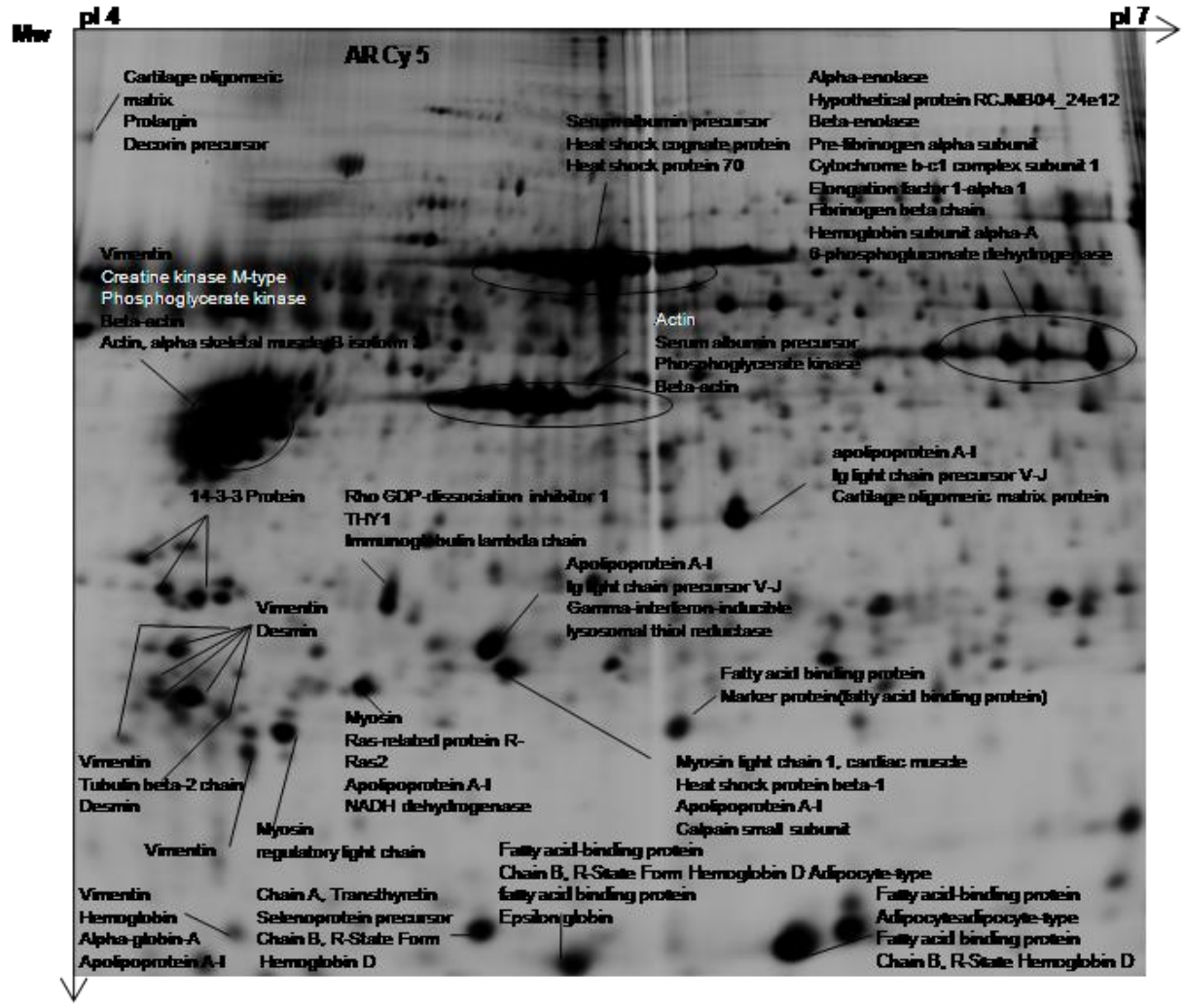


Figure 6.9: Proteome of chicken gastrocnemius tendon (white labels are used for clarity only). Table 6.1 lists and details the proteins identified from the tendon 2D DiGE gel, Figure 6.10 is the same gel with numbered labels. Separation was achieved by binding tendon lysate to Cy3 and Cy5 dyes. Proteins were separated over a pi range of 4-7 using 24cm IPG strip (GE Healthcare 17-6002-32) following reduction and alkylation steps. Following isoelectric focusing the proteins were separated in the second dimension on a 12.5% SDS-PAGE gel. Protein identifications were made from spots identified in the DiGE gels and matched from the preparative gels (Figures 6.7 and 6.8). Excised spots were trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates.

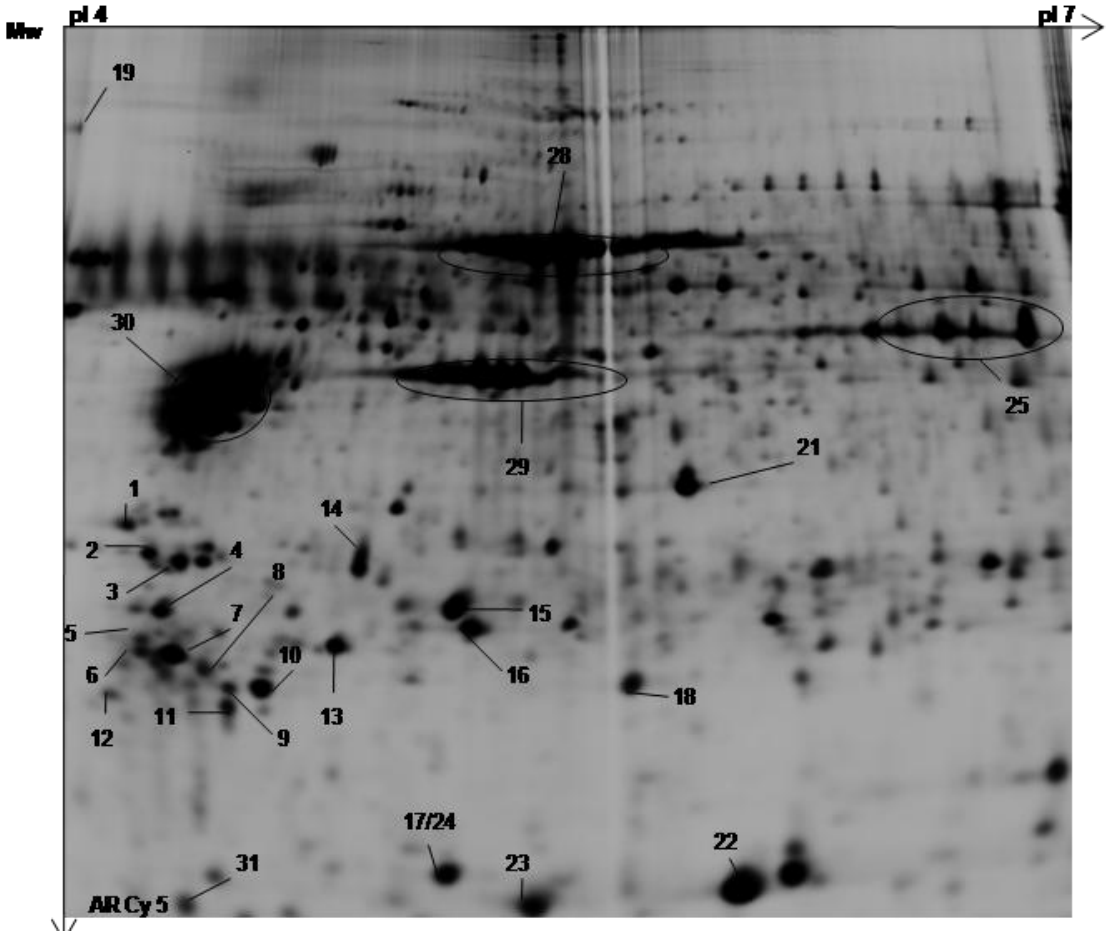


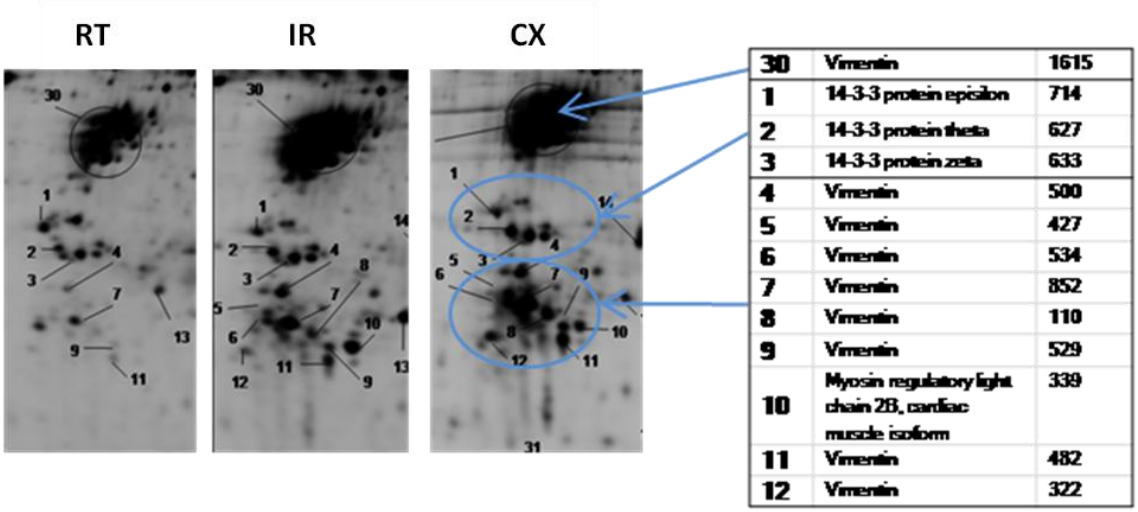
Figure 6.10: Proteome of chicken gastrocnemius tendon (as shown in Figure 6.9) with numbered labels that correspond to proteins listed in Table 6.1.

6.3.4 Proteins of interest

To determine whether there were any differences in the proteomes of the RT, IR and CX tendons visual inspection of the gels was undertaken as statistical analysis could be applied owing to absence of standards on the gels. Despite this two areas of difference between the RT gels and the gels of the IR and CX were clear and are detailed in Figures 6.11 and 6.12.

The entire left quarter of the gel is highlighted in Figure 6.11. The most intense protein spot / spot cluster is spot 30, identified with a very high score as vimentin. Spots 1,2 and 3 are identified as 14-3-3 proteins, of various isoforms and spots 4 – 12 at a lower molecular weight were also identified as vimentin (apart from spot 10). It is clear from the gels that the spots identified as vimentin, both in the major spot and at the lower molecular weights are

distinctly lighter in the RT gels compared to the IR and CX gels. Unfortunately the Cy3 gel for RT was gel 1 which as detailed early scanned at a lighter resolution, however even on this gel on which the CX sample was also ran, showed the vimentin spots were much more intense than on the RT gel. In Figure 6.12 Apolipoprotein A1 (ApoA1) was found in all 4 spots detailed in Figure 6.12, with mascot scores the highest in spots 15, 26 and 27.



30	Vimentin	1615
1	14-3-3 protein epsilon	714
2	14-3-3 protein theta	627
3	14-3-3 protein zeta	633
4	Vimentin	500
5	Vimentin	427
6	Vimentin	534
7	Vimentin	852
8	Vimentin	110
9	Vimentin	529
10	Myosin regulatory light chain 2B, cardiac muscle isoform	339
11	Vimentin	482
12	Vimentin	322

Figure 6.11: Significant area of interest is spot 30, showing a clear difference in expression between RT and IR and CX. The large mass of spots was identified as vimentin. The middle spot cluster was identified as 14-3-3 protein epsilon and the lower cluster as predominantly vimentin. Both the large top spot mass and the lower cluster of spots identified as vimentin are reduced in the RT.

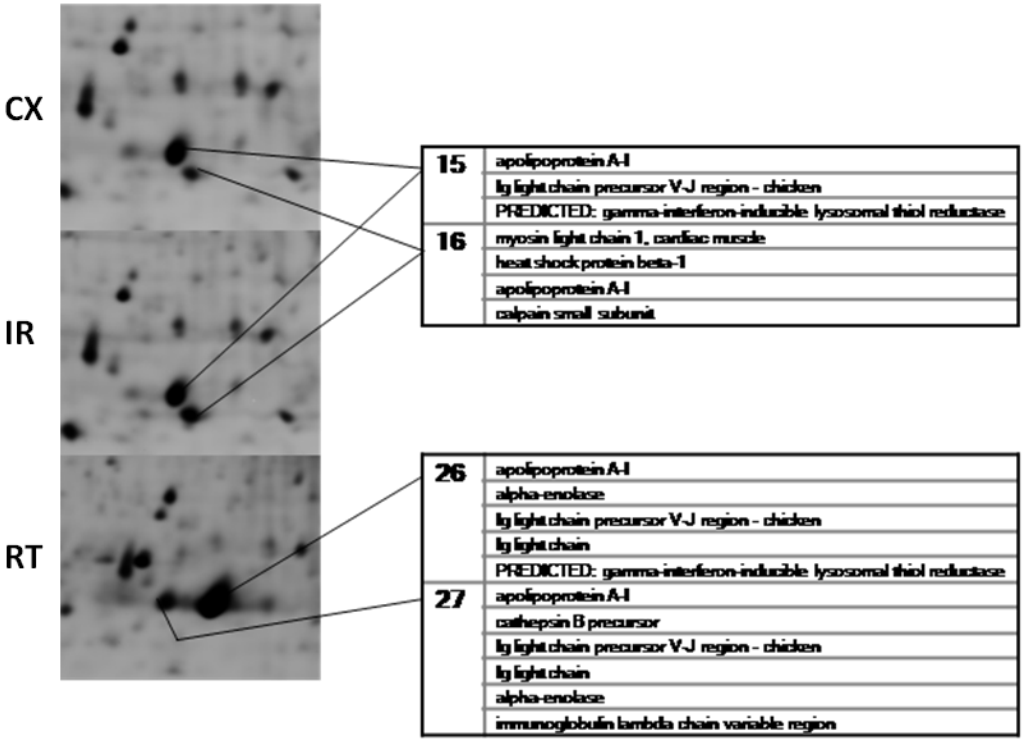


Figure 6.12: Area of interest and mascot results from the mid-section of the gel. Clear difference between the apo-AI spots between RT and CX and IR are evident.

Looking at the gene ontologies (GO) for the proteins identified in the tendon proteome, Figure 6.13 compared molecular function, cellular component and biological process, to give an overall view of the proteins identified with mass spectrometry, with an large number of the identified proteins functioning as binding proteins.

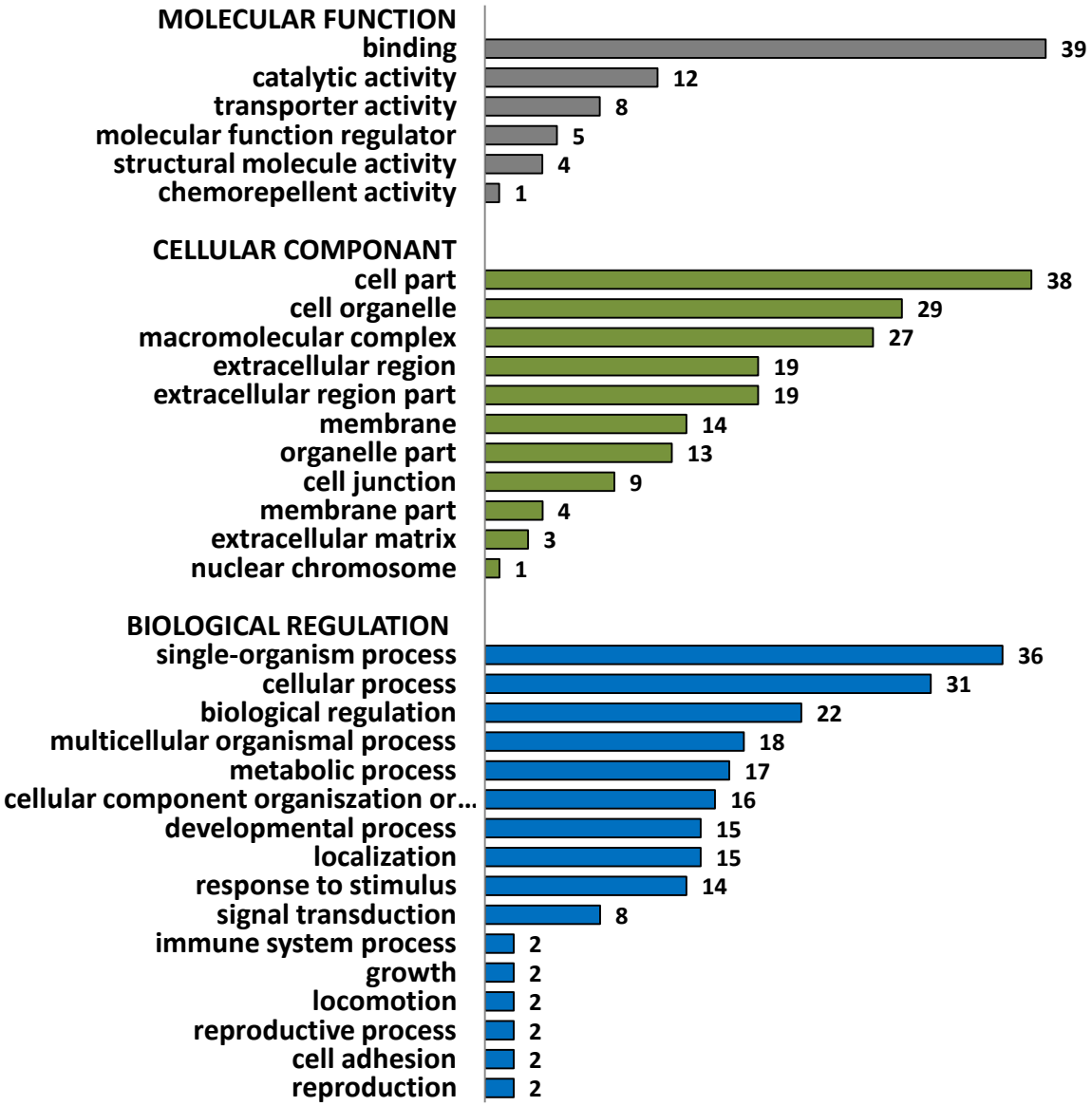


Figure 6.13: Gene ontology view of proteins identified in the tendon proteome (GO terms and data: UniProt.org). For all proteins identified from spots excised from the preparative gels, the GO terms were determined and compared using UniProt's GO tool for comparison of molecular function, cellular component and biological regulation of proteins.

6.4 Discussion

6.4.1 Proteomic methods targeting tendon proteins

Very few proteomic studies of tendons have been undertaken, and there are none reported in chickens. Of the proteomic studies available, comparisons are difficult because studies have been *in vitro* or used *in vivo* models of healing. Collagen, the main structural protein of connective tissue, is the most abundant protein in tendons forming 70-80% of the dry matter, with type I collagen forming 95% of the total collagen content and 5% consisting of type III and V collagens (Moussa *et al.*, 2008). Despite its abundance no collagen proteins were identified in any of the spots identified by mass spectrometry. This is due to the insolubility of collagen protein and a preparation buffer and homogenisation method that targeted only the soluble proteins. As a result it was the non-collagenous proteins that were identified in this study. Non-collagenous proteins are derived from the extra cellular matrix (ECM). The ECM of the tendon contains proteoglycans and their associated glycoaminoglycans chains and other non-collagenous proteins. The non-collangenous matrix proteins provide the interfibrillar links that bind the collagen fibrils one to another in a molecular bridge allowing force transfer (Moussa *et al.*, 2008).. For the purposes of this study it was the non-collagenous proteins which are of special interest, as it has previously been established that the GT of birds resistant to GT rupture contain relatively more collagen with collagen fibres showing better alignment and a higher degree of cohesion (van Walsum *et al.*, 1981). Other areas of proteomic research are hampered by the presence of highly abundant protein, so it is arguably preferable to have a method that by design and the nature of the target tissue results in the exclusion of the most abundant proteins.

The initial 2DE utilising the BioRad system with Coomassie staining was not successful, Figure 6.6 being a single good example of a gel produced using this method. Gels produced lacked repeatability the same samples producing vastly different results often with streaking or no result at all. The proteomic analysis of tendon is known to be problematic, even with the absence of insoluble protein such as collagen, the presence of highly anionic macromolecules causes

extreme charge density that can interfere with protein separations (Önnerfjord *et al.*, 2012; Little *et al.*, 2014).

Other studies have employed better methods to target this tissue, such as in Little *et al.*, (2014), where heating, pulverisation and sonication were used and trypsin digestion of the tendon protein took place *in situ*, resulting in 100% solubilisation of the protein before identification took place using quantitative two-dimensional liquid chromatography – tandem mass spectrometry. More proteins, including the insoluble proteins were therefore identified in this study. When investigating the literature, only Sato *et al.*, (2012), for a preliminary study, has targeted the tendon proteome using the DiGE method. The current study was successful in identifying DiGE as a suitable method for identifying and mapping proteins within a tendon proteome finding separation of the proteins to be very good. The preparative gels, from which the spots were picked however, were subject to the same limitations as the SDS-PAGE gels, and as such matching to the DiGE gels was limited, resulting in many DiGE spots not being identified. The difference in the results of these two methods using the same tendon samples, make it highly probable that protein concentration is also a factor, with the lower concentration used on the DiGE (a tenth of that used on the preparative gel) resulting in much better separation. Any future studies could utilise the alternate DiGE method of loading additional unbound protein (which would normally be loaded on to a preparative gel) alongside the Cy-bound protein onto a DiGE gel. This would allow for Coomassie staining after the DiGE gel had been scanned and spots picked from the original gel, thus avoiding any possible problems with a large protein concentrations and also spot matching.

6.4.2 The tendon proteome

As a preliminary study which excluded a Cy2 labelled standard, no statistical analysis could be performed yet it was clear that parts of the RT proteome differed with differential expression of a number of spots highlighted in Figures 6.11 and Figure 6.12. Figure 6.11 shows two areas with multiple identifications for vimentin: the large mass of protein labelled as spot 30 and a cluster of spots below it (spots 4-9 and 11-12). In both areas the vimentin spots are much smaller in the RT indicating reduced expression. These two areas flank a cluster of spots (1-3), which were all identified as various isoforms of the protein 14-3-

3, a class of highly conserved proteins involved in regulating signal transduction pathways, apoptosis, adhesion, cellular proliferation, differentiation and survival (Mhawech, 2005). Protein 14-3-3 does not differ in intensity between the different tendon groups, adding weight (in the absence of internal standard) to the differential expression of vimentin.

Vimentin is the major intermediate filament (IF) protein found in mesenchymal cells and is frequently used as a developmental marker of cells and tissues. Intermediate filaments represent the main cytoskeletal and nucleoskeleton systems found in virtually all vertebrate cells (Eriksson *et al.*, 2009). Vimentin sequences show a high degree of homology among species implying important and evolutionary conserved physiological roles. Vimentin is involved in several critical cellular processes related to the organization and regulation of proteins involved in adhesion, migration, and cell signalling (Eriksson *et al.*, 2009). Vimentin also has a role in the mechanotransduction of shear stress and studies on resistance in arteries has shown vimentin to be sensitive to mechanical stresses, including shear stress (Henrion *et al.*, 1997). This, together with what appears to be differential expression between the tendons in this study, points towards a possible role in the aetiology of ruptured GT. Desmin, also identified, alongside vimentin, in spots 4, 5, 7 and 9, is also a IF found at myotendinous junction (Tidball, 1992).

Heat shock protein was identified in two spots: 16 and 28. Spot 16 as detailed in Figure 6.12 showed differential expression between RT and CX and IR. Spot 28 is a large mass of spots that dominants all the gels but on visual inspection it does appear less intense in the RT compared to the other two groups. Heat shock proteins are a group of proteins synthesised in response to physical, chemical or biological stresses, including heat exposure (Gu *et al.*, 2012). They are molecular chaperones responding to stress related events in a variety of organs. Heat shock proteins protect against environmental stresses and are considered important for adaption to environmental changes (Otaka *et al.*, 2009). Heat shock proteins protects against ischemic and reoxygenation mediated injury and inhibits apoptosis induction (Cohly *et al.*, 2002). Jielile *et al.*, (2011) found that heat shock protein increased in expression between early motion rabbits and immobile rabbits post gastrocnemius tenotomy, and attribute this to the

increase in cellular stress. Heat shock B1 is also believed to be a collagen binding chaperone involved in the maturation of collagen (Jielile *et al.*, 2011).

As a vascular tissue, proteins from blood bathe tendon or ligament extracellular matrix in intravascular and extravascular interstitial fluid, but are not typically interrogated as part of tendon or ligament extracellular matrix. Nonetheless, they may play a role in tendon and ligament homeostasis and disease and may be enriched in different tendon and ligament structures (Little *et al.*, 2014). Albumin is present in serum at concentrations of 20–50 mg/mL, and is one of the most abundant blood proteins, thus as a conservative estimate any blood, serum or amyloid protein present in a vascularised tissue at similar or higher levels than albumin would be expected to be preferentially enriched within the tissue (Little *et al.*, 2014). In the current study albumin was identified in spots 28 and 29. Spot 28 is a large band rather than a spot with albumin forming the main protein, with a high mascot score of 1557, and as such it is clear that blood components form part of the tendon proteome in this study. Differences in blood derived proteins were expected between RT and other tendons given the degree of tissue haemorrhage seen in the RT during preparation (Figure 6.3.a.) and spot (band) 28 is larger in the RT tendons compared to the RT and IR tendons. Jielile *et al.*, (2011) in a study of post tenotomy healing in rabbit GT, found albumin was increased in expression in the immobile group compared to the early motion group. Should the current study be repeated in addition to the inclusion of Cy2 bound internal standards to allow for statistical analysis, when comparing groups the quantitative differences in the GO terms relating to blood/plasma between two or more groups in relation to albumin could be applied as described in Little *et al.*, (2014) to give an indication as to what blood proteins are present at enriched concentrations within the tendon. In addition to albumin, transthyretin, haemoglobin and fibrinogen were all identified in the tendon proteome.

Of the spots shown in Figure 6.12 two large spots, 26 and 27 in the RT appear absent in the IR and CX tendons. Apolipoprotein A1 (ApoA1) was found in all 4 spots detailed in Figure 6.12, with mascot scores the highest in spots 15, 26 and 27. Spots 26 and 27 are large spots and also contain α-Enolase, a protein not identified in the IR and CX tendons. Apolipoprotein A1, a major component of high density lipoprotein (HDL) in plasma, is involved in lipid transport and

metabolism. Apolipoprotein A1 has anti-inflammatory properties (Kravitz *et al.*, 2005) and in avian species ApoA1 is expressed in numerous other tissues other than the liver, the primary site of synthesis in mammals (Doherty *et al.*, 2004). ApoA1 is thought to act as a local lipid transporter (Doherty *et al.*, 2004) and its presence in the RT could be due to the increase in cellular debris, brought about by the damaged tissue. α-Enolase, a highly conserved glycolytic enzyme, is a multifunctional protein that serves as a plasminogen receptor on the surface of a variety of haematopoetic, epithelial and endothelial cells playing an important role in the intra-vascular and pericellular fibrinolytic system. Additionally it functions as a heat shock protein, binds cytoskeletal and chromatin structures playing a crucial role in transcription and a variety of pathophysiological processes. It also increases in a variety of auto- immune diseases (Pancholi, 2001). Enolase enzymes have not been identified as being differentially expressed in other studies of the tendon proteome.

Annexin is a calcium-dependent phospholipid-binding protein that plays a role in the regulation of cellular growth and in signal transduction pathways and is also involved in a number of biochemical processes, including cell proliferation, ion channel activation, and cell– cell interactions (Jielile *et al.*, 2011). Annexin has been identified as being associated with tendinopathy and is known to be involved in the proposed mechanisms of tendinopathy, including hypoxia or apoptosis (Little *et al.*, 2014). Annexin was identified in this study, but it was unclear whether there were any differences between the groups. Annexin was found to be higher in the early motion group than the immobilised group in Jielile *et al.*, (2011), the authors postulating whether the presence of annexin A2 in the samples of the early motion group could facilitate proliferation of tendon cells and an increase in the circumference of collagen fibers and as such be involved in many normal and pathological processes which accelerate GT healing (Jielile *et al.*, 2011).

Other proteins that appear differentially expressed between groups include cathepsin B precursor, found exclusively in the RT group. Cathepsin B is a papain family cysteine protease that is normally located in lysosomes where it is involved in maintaining normal metabolism of cells. This protease has been implicated in many pathological conditions including tumour formation, osteo- and rheumatoid arthritis (Yan & Sloane, 2003). Myosin light chain 1 was

identified in spot 16 on the IR and CX gels but appeared absent in RT gels. Myosins are a large superfamily of proteins that interact with actin, hydrolyse ATP to produce movement (Sellers, 2000).

To expand this investigation beyond this preliminary study the protocol would benefit from a number of changes in addition to the ones already described. A more localised approach, to focus on the fibrocartilage attachment area at the hypotarsus where the GT rupture occurs may yield more informative results. Further DiGE work should involve more replication and the use of internal standards that would allow the associated DiGE software to determine which proteins are statistically different between groups, and this may allow for subtle differences between the gels to be identified. With a view of identifying proteins that increase the risk of GT rupture, concentrating on the IR and CX tendons may be preferable, so as to exclude proteins present as a result of the traumatic tissue damage caused by GT rupture. As described earlier the adoption of a protein extraction method that achieves 100% solubilisation of the tendon protein could be advantageous, as would a comparative study to determine whether leaving the insoluble protein out of the study does in fact enhance protein identification of less abundant proteins. The results of this study, as well as identifying problems with and improving the described method does at this preliminary stage suggest that differentially expressed proteins, particularly vimentin play a role in the aetiology of GT rupture in broiler chickens.

Chapter 7

General Discussion

The aims of this thesis were outlined in Chapter 1 and the results of the work discussed in each of the chapters that followed. This chapter will summarise and discuss the most significant of these findings and elaborate on possible areas of future study.

Chickens are the most populous farm animal in the word, with an estimated worldwide flock of 21 billion (Blake & Tomley, 2014). The human world population is growing, there is a demand for more affordable protein rich foods in regions with increasing population and this is a major driver for this change (Lassaletta *et al.*, 2014). Expansion of the poultry industry is predicted to continue for at least 30 years with Africa and Asia accounting for the most growth (Blake & Tomley, 2014). Management practices, biosecurity, nutritional improvements, R&D investment and advancements in genomics enable the poultry industry to maintain and grow their industry in a challenging economic and microbial environment. Commercial poultry production is only possible with the support of effective pathogen control, chemoprophylaxis and vaccination (Blake & Tomley, 2014). The trend of increased consumer demand in an era where antimicrobial prophylaxis and treatment is under close scrutiny requires novel strategies to maintain flock health and combat disease. Bacteria and viruses are the major variables, constantly evolving to cause antimicrobial resistant pathogens that lead to outbreaks of disease that are difficult to treat. Low level, sub-acute disease with non-specific, often undiagnosed causes can greatly affect bird health and growth, and impact enormously on productivity.

The concept of this research was borne out of a need to identify ways in which the early IIR of the chicken could be measured and assessed. As an early non-specific response the APR is a highly suitable target for biomarker investigation. While the immune system of the broiler chicken has been a long standing area of research interest, translating this interest into actionable methods that can be applied to diagnostics or as a tool for selection have been shortcoming. As quantifiable mediators of the APR, APP have been studied alongside disease research in chickens, however the limited availability of anti-sera and ELISAs has resulted in a lot of information on only a few APPs.

Many of the studies follow the same approach, measuring APPs in specific disease models or injecting birds with LPS. While these deliver informative results initially, the publications regarding APPs in chickens over the last 10 years have offered no new insights into identifying and confirming other APPs in chickens or methods to measure them. No studies have measured any APP in normal chickens from commercial farms and none have sought to measure APPs during generalised poor growth and performance as a method to characterise general chicken robustness. For APPs to be used diagnostically and as selection tools then this information is needed in order to address these data deficient areas.

The lack of baseline data was addressed with the first study measuring PIT54 and Cp in healthy and culled birds from a commercial flock. Baseline data has previously been derived from the control groups of challenge studies and therefore not representative of normal populations. The results for the healthy birds were 12 and 9 fold lower serum concentrations for PIT54 and Cp respectfully than the average reported control results in published reports. It would appear that healthy chickens on commercial units have lower baseline concentrations of PIT54 and Cp than what would be deduced from the published data. The low concentration of PIT54 and Cp was also seen in a number of samples and studies that were investigated during this research. As minor APPs with small fold changes, the concentrations of these APPs are low and sometimes undetectable. The PDP oxidation method for measuring Cp was problematic owing to the relatively quick rate of oxidation observed, which limited the number of samples measured at any one time and also increased the inter-assay CV to over 20% which is undesirable.

While measuring other more moderate and major APPs may reveal more information, as a starting point this was informative. This area would benefit from being revisited, measuring a wider variety of APPs on a larger numbers of samples. This type of study will always be difficult owing to the fact that producers are unwilling to sacrifice healthy birds. Moving sampling to the slaughterhouse would overcome this issue, yet transport stress is known to increase APPs in pigs (Piñeiro *et al.*, 2007) and as such would not be reflective of normal concentrations.

Measuring APPs in broiler chickens at differing ages is suggested for future studies. Measuring APPs by both immunoassay and quantitative proteomics in chickens challenged with re-used litter from day 12 to 22 found that AGP increased significantly from day 12 to 22 in all chickens, irrespective of challenge (O'Reilly, 2013). The present study found SAA and Hpx to increase over the same time period, although not significantly. The plasma concentrations of the other APPs measured Ovt, Cp, Apo-AI, Ttn and PIT54 did not change overtime. Further studies to fully characterise the changes in APP expression over the normal production life of broilers and broiler breeders would provide valuable reference material.

The early part of this thesis looked at the effect of gait score on APP concentrations and measured APPs in birds culled from commercial farms for lameness. Lameness is an issue for the broiler industry in terms of animal welfare and economic loss, however there are 15 separate conditions in broiler chickens that can result in lameness (Neeteson, 2010). Many of the culled lame birds had APP profiles comparable to the healthy birds and although GS3 birds were found to have significantly higher PIT54, Cp and Ovt than sound GS1 birds, the multiple aetiologies of lameness, including those known to not cause elevations in APPs, namely tibial dyschondroplasia, make using APPs as a biomarker for lameness difficult. The significant association between bird weight and Cp concentrations across all birds is an interesting but unexplainable finding. There are a number of possible reasons that could account for this finding, the most basic being that bigger birds produce more Cp, with increased dietary intake enabling increased synthesis of Cp and increased growth.

While not strictly relevant to the poultry industry, the higher 5mg/kg dose of meloxicam, significantly increased plasma Ovt concentrations. This suggests that the NSAID had a deleterious effect on the gastrointestinal tract which resulted in the translocation of bacteria and a resultant APR, as described in humans and rodent models (Tugendreich *et al.*,2006). This finding is highly relevant to backyard and pet chickens that receive meloxicam off licence and caution should be applied to prescribing dosages above 2mg/kg.

These early studies uncovered a number of difficulties with the competitive Ovt ELISA, prompting the development, validation and subsequent use of an Ovt RID assay. Unlike the competitive ELISA the RID assay had good CVs, was easy and straightforward to use and more economical. This assay can also be undertaken in the most basic of labs with no need for specialist equipment. Developing an automated turbidimetric assay with the same antibody would increase throughput of this assay enabling many more samples to be measured and allowing full characterisation of Ovt in broiler chickens to be made. Further validation of this assay and antibody was achieved in Chapter 4, using quantitative proteomics, where the relative concentrations of three of the four Ovt peptides, found were significantly correlated with the results of the RID assay. Table 7.1 summarises the results of all the studies that have quantified an APP in this thesis. Results are listed by APP with the method of measurement detailed also. Table 7.2 summarises chicken APPs providing an updated classification of APPs in this species.

APP (method)	Chapter 2								Chapter 4		Chapter 5		
	Culled bird study		Gait score study		NSAID		Coccidiosis study		Quantitative proteomics		Gut litter challenge study		
	Healthy	Culled	GS1	GS3	Saline	NSAID	Control	Challenged	Non-acute phase	Acute phase	day	Control	Litter challenged
Ovt (ELISA) g/L			2.15 (0.73)	2.44 (1.73)*	2.20 (0.75)	2mg/ml = 2.05 (0.79) 5mg/ml = 2.75 (2.15)*	2.165 (0.1435)	*E. tenella* = 3.86 (1.56)* *E. tenella* & *E. coli* =4.70 (1.54)*					
Ovt (RID⁺) g/L									1.34 (0.36)	Highly acute phase = 4.31 (1.8) Acute phase = 1.63 (0.55)	12	1.49 (0.26)	1.55 (0.34)
											15	1.36 (0.29)	1.28 (0.25)
											18	1.34 (0.21)	1.29 (0.32)
											22	1.39 (0.14)	1.56 (0.26)
Cp (PPD) g/L	0.00 (0.03)	Lame = 0.43 (0.91) Stunting = 0.07 (0.07)*	0.72 (0.28)	0.98 (0.68)**	2.20 (0.75)	2mg/ml = 0.90 (0.67) 5mg/ml = 0.86 (0.50)	0.002 (0.007)	*E. tenella* = 0.15 (0.14)* *E. tenella* & *E. coli* =1.67 (2.09)**					
Cp (ELISA) g/L											12	0.13 (0.07)	0.14 (0.02)
											15	0.13 (0.01)	0.17 (0.07)
											18	0.11 (0.06)	0.16 (0.05)
											22	0.21(0.07)	0.19 (0.06)
PIT54 g/L	0.10 (0.01)	Lame = 0.19 (0.29) Stunting = 0.62 (1.33)	0.10 (0.03)	0.12 (0.023)*	2.20 (0.75)	2mg/ml = 0.11 (0.03) 5mg/ml = 0.10 (0.03)							
SAA (ELISA) ng/µl									0.05 (0.04)	Highly acute phase = 0.43 (0.01)* Acute phase = 0.24 (0.10)	12	0.66 (0.28)	0.89 (0.20)
											15	1.28 (0.99)	0.79 (0.65)
											18	1.15 (0.62)	1.45 (0.71)
											22	2.31 (0.90)	1.89 (1.22)
AGP (RID) µg/ml									247.79 (38.28)	Highly acute phase = 1344.32 (583.02)* Acute phase = 880.68 (154.98)*	12‡	155.45 (68.37)	120.19 (69.0)
											15‡	172.88 (22.95)	193.31 (41.49)
											18‡	198.08 (92.71)	210.94 (55.34)
											22‡	217.68 (50.76)	343.45(184.42

Table 7.1.

Table 7.1: Summary of all the APPs measured throughout thesis over a number of different studies, with chapter containing full details of the studies and methods of measurement indicated. Value shown is the mean (±SD). Where significant differences relative to the control/healthy were observed this is indicated

(*p=<0.05, **p=<0.01). [+] Developed in Chapter 3. [‡]While no significance was detected between challenge and control samples. The AGP was found to increase significantly over time in both control and challenged.

Acute phase protein	Classification	Method	In agreement with literature?
Serum amyloid A	Major positive	Immunoassay & Quantitative proteomics	✓
Alpha-1-acid glycoprotein	Moderate positive	Immunoassay	✓
Ovotransferrin	Moderate positive	Immunoassay & Quantitative proteomics	✓
PIT54	Minor	Biochemical reaction	✓
Ceruloplasmin	Minor	Immunoassay & Biochemical reaction	✓
Apolipoprotein-AI	Negative	Quantitative proteomics	**Novel finding**
Apolipoprotein AIV	Not an APP in chickens*	Immunoassay & Quantitative proteomics	**Novel finding**
Haemopexin	Minor positive	Quantitative proteomics	X
Transthyretin	Negative	Quantitative proteomics	**Novel finding**
Fetuin	Negative	Quantitative proteomics	**Novel finding**
CRP	**Not an APP in chickens**		

Table 7.2: An updated tabl classifing APPs in chickens. Both immunoassays and quantitative proteomics were used in a series of experiments on experimental studies on gait score, NSAIDs study, Coccidiosis study and a reused litter challenge study and also more general investigations such as that undertaken on culled birds. A quantitative proteomics approach was also used to identify and relatively quantify APPs between acute phase groups.
*Further work to fully characterise needed.

While APPs such as Ovt, PIT54 and Cp are covered widely in the literature, there are many reputed APPs that have not been studied in chickens, and this is because of limited anti-sera availability and the reduced chances of successfully appropriating anti-sera from other species. To overcome these issues and identify and characterise the full spectrum of APPs in chickens, a quantitative proteomic approach was taken. The APPs SAA and CRP were particular targets owing to the previous work reporting SAA as a major APP in chickens (N Upragarin, 2005) and because of C-reactive protein's prominent position as the major APP in humans, dogs and pigs and subsequent inclusion in a number of publications on chickens. As the early work on these proteins was unsuccessful (not included in thesis) it was clear that enrichment steps would need to be undertaken on both of these proteins prior to shotgun analysis. Molecular weight fractionation was successful in enriching acute phase serum sufficiently to identify more SAA peptides but despite both molecular weight enrichment and the affinity chromatography, CRP was not identified. Relaxing the search criteria resulted in one peptide being matched to CRP but this was not sufficient for an identification to be made. From these results it is clear that CRP is not an APP in chicken. Further steps to validate these findings could be undertaken, but the given the sensitivity of the MS and the enrichment steps undertaken and the number of samples this was applied to, it is strongly suspected that CRP is not present at all at a protein level chickens. It is therefore frustrating to see multiple papers using non-specific, commercially acquired ELISA kits to measure "CRP" in chickens. While there is a CRP gene present, it would appear that it does not result in the expression and secretion of CRP. A recent paper using qPCR to look at expression of immune genes in the spleen of chickens infected with parasite *Ascaridia galli,* targeted CRP finding expression to increase less that 2 fold at one time point, the authors noting that 'few reports exist on chicken CRP' (Dalgaard *et al.*, 2015).

The APPs Apo-AI and Ttn were confirmed as negative APPs in this study. There are no reports that detail these APPs in chickens, the findings here confirm that they are present at lower quantities in HAP and AP birds during at APR and as such classified as negative APPs. Negative APPs and other biomarkers are often not as 'popular' as positive ones. An increasing biomarker has an infinite possibility, whereas a negative one cannot get any less than zero. However the

decrease of constitutive proteins during an APR is a significant event that should draw more interest than it currently does. Often referred to as “acute booster reactants” to highlight the cascade of helpful events (Ingenbleek & Bernstein, 2015) generated by their reduced synthesis, increased breakdown or loss of these proteins, the decrease in their concentration has to confer a benefit to the animal.

It has been postulated that the decrease in the major negative APP albumin during an APR is to allow for the unused pool of amino acids to instead be used to generate positive APP and other important mediators of inflammation (Cray *et al.*, 2009). This is possible as the fractional synthesis rate of total liver proteins (stationary and exported), equals approximately 25% of the liver protein content daily (Barle *et al.*, 1997). Further still, in humans the amount of albumin synthesised per day (absolute synthesis rate) is calculated to be 109 ± 21 mg per kg of body weight (Barle *et al.*, 1997). While not completely comparable, if this was to be extrapolated for a 2kg chicken, it would equate to over 1.5g of albumin in a week during homeostasis. During an APR, the decrease of albumin and negative APPs, apo-A1 and Ttn for the partitioning of amino acids towards synthesis of positive APPs and other mediators of the immune system, if insufficient to meet the needs of the APR, results in redirection of other energies towards the immune system which results in the problem surrounding the ‘costs’ of the immune system that were discussed in Chapter 1. Focusing on the negative APPs may prove insightful when considering this issue, the negative APPs should perhaps be considered “constitutive APPs”. If proteins such as albumin, apo-A1 and Ttn provide the building blocks of the early IIR, ensuring these plasma proteins remain at optimal levels in the healthy bird may reduce the mounting costs associated with an effective APR.

Having established the value of using quantitative proteomics, this method was employed to identify proteins that were differentially abundant between three acute phase pools. The advantages of using quantitative shotgun methods for identifying and measuring proteins cannot be over stated. It allowed a broad approach to be taken that moved away from the ‘looking for what you’re expecting to find’ approach that had previously dominated the APP work in chickens. Figure 7.1 is a well circulated cartoon that illustrates this point well.

Acute phase proteins are known to behave differently between species so looking for the same targets across species is not a good approach.

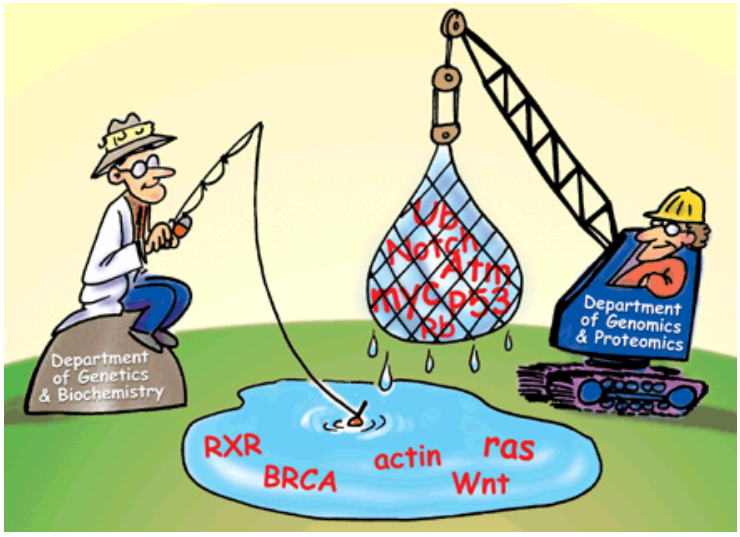


Figure 7.1: Shotgun proteomics: a more effective way of identifying biomarkers (Image: Fields, 2001).

The data collected during the shotgun proteomics, namely the quantotypic peptides for each protein and their retention times, can be used again and will save considerable time on future studies. As the data accumulates for chickens and technologies develop and preparative methods simplify, quantitative proteomics will become more widespread. It is an apt method for measuring a large number of proteins on a limited number of samples.

Many of the proteins targeted for quantitative measurement were differentially abundant between the HAP and NAP or the HAP and the AP groups. It is not desirable to have a biomarker that shows massive increase or decrease between the HAP group and the other acute phase groups. On a farm the HAP birds would be evidently very ill or dead, negating the need for a biomarker! Biomarkers need to identify birds that are not obviously unwell or infected. The protein cathepsin looks to be the most promising protein identified as it is only protein that showed a significant difference between the NAP and the AP. None of the APPs targeted showed a significant difference between these two AP groups.

Biomarkers of intestinal health were targeted in this study. The intestinal health of broilers is a large and important area of research because of the important

role the gastrointestinal tract has in nutrient absorption, growth and maintaining the overall health of the bird. Plasma biomarkers for intestinal health and function would provide a non-invasive measure that, used in conjunction with other parameters, could aid in many areas of poultry health and nutritional research. Apo-AIV was identified as a protein of interest due to its clear differential expression on 2DE gels and its reported wide range of functions. An apo-AIV ELISA was successfully developed and validated. This ELISA was used to measure apo-AIV in HAP, AP and NAP samples and the intestinal challenge study. Apo-AIV was not significantly associated with the APR. This protein was also measured using quantitative proteomics. Apo-AIV in the intestinal challenge study was not significantly different between the challenge and control groups at any time point, however apo-AIV concentrations were significantly different over time for the control group (when measured using the ELISA) and the challenge group (when quantified using quantitative proteomics). The development of the apo-AIV ELISA means that this assay can be applied to a wide variety of studies where the behaviour and function of apo-AIV in chickens can be fully characterised. The protein villin, previously identified as a differentially expressed protein in the intestine of birds challenged with reused litter was found not to be present in the plasma of chickens. The introduction of the re-used litter was not sufficient to elicit an enteric disease that would cause villin, an intestine specific protein to move into the plasma. Villin is unlikely to be a suitable biomarker for intestinal health in chickens. A proteomic approach was also undertaken to evaluate the incidence of tendon rupture. The chicken GT proteome had not been studied previously and given the mixed aetiology and breed associated differences in incidence of GT rupture it was a good proteomic target. Because of the fibrous nature of tendon, it was a difficult tissue to work with and any future work would benefit from using liquid nitrogen to powderise the tendon before suspension in buffer. The inclusion of a Cy2 standard would also allow DeCyder software to identify spots that are statistically different in abundance.

This study has aimed to utilise samples and target tissues that closely reflect the normal production environment. Using tissues from naturally occurring disease (tendons) and realistic models of microbial challenge that affect growth (re-used litter), mean that when biomarkers are identified and later validated, they are

readily applicable to the poultry industry. It is relatively easy to identify differentially expressed proteins between samples, whether these translate into a useful biomarkers depends on a number of factors. Having the correct samples and study design from the outset makes the path from proteomics to biomarker development to industry application clear.

Moving forward, suggested areas for future work include focusing further on lipoproteins. SAA, apo-AI and apo-AIV have revealed themselves to be proteins of interest in this study and while SAA is difficult to work with it is the only major APP in chickens. Moving quantitative methods of APP measurement from the laboratory to the farm is also an area of future development. The development of lateral flow systems enabling quick on-site measurement of APPs with small volumes of blood would open up the field of APP biomarkers considerably. While it is recognised that relying on single trait criteria to select for improved immune competence has adventitious results, multi-trait selection criteria still requires measureable parameters of the early IIR and APP are suitable targets. While referring to APPs it must be considered that APPs are a collection of highly variable proteins, whose only commonality is that they are secreted by the liver and change in serum concentration during an APR. They have wide ranging functions and should, as possible selection parameters for breeding programs, be considered separately, as they are likely to have considerably different potentials as selection targets.

7.1 Conclusion

The results in this thesis have contributed novel and interesting information on the APR in chickens. Apo-AI and Ttn are both negative APP and SAA appears to be the only major positive APP present in chickens. The moderate APPs Ovt and AGP are readily measureable and good targets on which to focus future studies and developments. The evidence from this work reveals CRP not be an APP in chickens and is likely not present at a protein level during both homeostasis and during an APR. Quantitative shotgun approaches were used to identify proteins that were differentially abundant as a result of the APR and this lead to a targeted measurement of a number of potential biomarker targets, of which cathepsin appears the most promising.

An anti-Ovt antibody was produced using the egg derived antigen that works successfully in a developed and validated RID immunoassay. An ELISA for measuring apo-AIV was developed and validated and applied to a number of studies. Apo-AIV in chickens appears not to behave as an APP and while the results are mixed, apo-AIV does appear to be affected by the introduction of re-used litter. Villin was not identified in the serum or plasma of chickens and as such an unlikely biomarker for intestinal disease. The tendon proteome was evaluated in ruptured and normal tendons, where the application of the DiGE revealed a number of differentially expressed proteins.

The information presented in this thesis has expanded the knowledge of chicken APPs and developed further methods of measurement that will allow the study of APPs in chickens to continue beyond this work. Using the latest proteomic technologies to identify and quantify APPs and investigate other biomarkers is, for this species novel and has resulted in a long overdue update on APPs in chickens.

Appendices

Appendix A

Publication

O'Reilly, E. L. & Eckersall, P. D. (2014) *Acute phase proteins: a review of their function, behaviour and measurement in chickens* World's Poultry Science Journal **70** (1) 27 – 44

doi:10.1017/S0043933914000038

Acute phase proteins: a review of their function, behaviour and measurement in chickens

E.L. O'REILLY* and P.D. ECKERSALL

Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Bearsden Road, Glasgow, G61 1QH, United Kingdom
***Corresponding author: e.o'reilly1@research.gla.ac.uk**

This review brings together and consolidates the large amount of research on acute phase proteins (APPs) that has been undertaken in chickens. Acute phase proteins are secreted from the liver as a result of inflammation or infection that can be measured in plasma. They have been well-characterised in other farm animal species and have been measured in a wide variety of poultry research areas. The acceleration in chicken APP research is in response to increased interest in ways the immune responses of the chicken can be measured and compared during infection or environmental or nutritional changes. All APPs that have been identified and characterised in chickens are described in the following review and their responses during infection discussed. The APPs are tabulated with basal values and classification to provide a comparative and useful reference. The ways APPs can be measured in chickens and the assays available are also described. This review will detail the functions of the positive APPs in chickens and their behaviour during an APR.

Keywords: chicken; acute phase protein; biomarker; infection

Introduction

The acute phase response (APR) is an early and non-specific systemic reaction of the innate immune system to local or systemic disturbances caused by trauma, infection, stress, surgery, neoplasia or inflammation, the goal of which is re-establishment of homeostasis and healing (Gruys *et al.*, 2005; Cray *et al.*, 2009; Eckersall and Bell, 2010). At the site of infection or location of tissue injury, pro-inflammatory cytokines and chemokines are released (Gruys *et al.*, 2005). Cytokines and chemokines are protein and peptide mediators secreted by cells which play a key role in immune and inflammatory responses through activation and regulation of other cells and tissues (Wigley and Kaiser, 2003). These inflammatory mediators initiate and modulate the

World's Poultry Science Journal, Vol. 70, March 2014
Received for publication February 5, 2013
Accepted for publication October 7, 2013

APR, diffusing into the extracellular fluid and circulating in blood. Cytokines activate receptors on different target cells leading to a systemic APR and resulting activation of the hypothalamic-pituitary-adrenal axis, reduction in growth hormone secretion, and a number of physical changes clinically characterised by pyrexia, anorexia and catabolism of muscle cells (Gruys *et al.*, 2005). Pro-inflammatory cytokines and chemokines together with nitric oxide and glucocorticoids activate hepatocytic receptors in the liver with resultant changes in protein synthesis and secretion. As a result within the first few hours of an APR, there are measureable changes in the concentration of several plasma proteins referred to as acute phase proteins (APPs) (Gruys *et al.*, 2005).

The plasma concentrations of pro-inflammatory cytokines which increase within hours of the initiating stimulus are rapidly cleared from the circulation. Acute phase protein plasma concentrations are detectable for longer periods and are often proportional to the severity of the stimulus. As such APP measurements are widely used as disease biomarkers and for prognostication in human and veterinary medicine and are increasingly used in varied areas of research.

Acute phase proteins can increase or decrease during an APR and these are termed positive APPs or negative APPs respectively. Albumin is the most abundant plasma protein and represents a major negative acute APP. Due to selective loss by the renal and gastrointestinal systems or decreased hepatic synthesis, plasma albumin concentration decreases during an APR. In chickens, albumin concentrations decrease to 50 - 75 % of normal concentrations (Grieninger *et al.*, 1986; Adler *et al.*, 2001). Positive APPs are further classified as minor, moderate or major APPs according to the magnitude and duration of increase during the APR. Major APPs increase 10 - 1000 fold, moderate APPs increase 4 - 10 fold and minor APPs represent those with only slight, 2 - 3 fold increases.

Major APPs tend to increase markedly within the first 48 hours of the triggering event and decline rapidly due to a short half life. Moderate and minor proteins tend to increase more slowly, peak at 2-3 days and have more prolonged duration (Eckersall and Bell, 2010). *Table 1* details the chicken APPs documented to date and, using published results, the basal levels and classification of minor, moderate or major. As well as the liver, APPs can also be synthesised in other tissues and organs. Although extra-hepatic expression of APPs does not account for the origin of the majority of APPs the contribution that other tissues make can be locally significant and furthermore, the expression pattern of an APP can vary between tissues.

APPs are involved in many crucial metabolic and immune pathways and have roles that include scavenging extracellular haemoglobin, iron and free radicals and direct antibacterial and antiviral activity. The response of APPs to various pathogenic challenges and other disruptions in homeostasis varies between species. APP profiles from both companion and farm animal species have been well documented, and in many veterinary species these clinical parameters are used diagnostically and for understanding the pathogenesis of important diseases (Petersen *et al.*, 2004; Eckersall and Bell, 2010).

Table 1 Basal levels of chicken APPs and their described behaviour during an APR.

APP	Abbreviation	Normal serum concentrations	Reference	Classification
Albumin	Al	11.08 ± 1.34 g/L 20 g/L (Plasma) 15 g/L (Serum)	Inoue *et al.*, 1997 Hrubec *et al.*, 2002 Hrubec *et al.*, 2002	Negative
α1-acid Glycoprotein	AGP	161.8 ± 25.8 mg/L 228 ± 20 mg/L	Inoue *et al.*, 1997 Takahashi *et al.*, 1998	Moderate
Ceruloplasmin	CP	30 mg/L	Disilvestro and Harris, 1985	Minor/Moderate
Fibrinogen	FB	0.99 (± 0.11) g/L 3.379 (± 0.485) g/L 2.8 (± 0.05) g/L	Georgieva *et al.*, 2010 Nazifi *et al.*, 2010 Amrani *et al.*, 1986	Minor
Fibronectin	FN	0.31 (± 0.4) g/L	Amrani *et al.*, 1986	Minor
Haemopexin	HX	0.15 g/L	Buyse *et al.*, 2007	Minor
Mannan binding lectin	MBL	0.4 - 37.8 mg/L (mean 5.8 (± 4.0) mg/L) Over 10 weeks old	Laursen and Nielsen, 2000	Minor
Ovotransferrin	OVT	0.90 ±0.06 g/L 1.18 (± 0.13) g/L	Xie *et al.*, 2002b Rath *et al.*, 2009	Minor/Moderate
PIT54	-	0.100 (± 0.020) g/L 1.2 g/L 0.09 g/L 0.05 g/L	Georgieva *et al.*, 2010 Millet *et al.*, 2007 Nazifi *et al.*, 2011 Nazifi *et al.*, 2010	Minor
Serum amyloid A	SAA	0.15 ± 0.02 mg/L 0.166 ±0.17 mg/L 1.590 ± 0.0041 mg/L 1.56 ± 0.13 mg/L	Alasonyalilar *et al.*, 2006 Sevimli *et al.*, 2005 Nazifi *et al.*, 2010 Nazifi *et al.*, 2011	Major

Acute phase proteins

SERUM AMYLOID A

Serum amyloid A (SAA) is a major vertebrate APP and in most species is the most sensitive protein of the APR. In mammals SAA can be induced from resting levels by more than 1000 fold, implying an important beneficial role in host defence (Eriksen *et al.*, 1993; Uhlar and Whitehead, 1999). In mammals, multiple SAA genes and proteins which are strongly induced during an APR have been described (Uhlar and Whitehead, 1999; Upragarin *et al.*, 2005). In contrast only one SAA gene has been identified in the chicken genome (Ovelgönne *et al.*, 2001).

Modulating lipoprotein transport and metabolism during an APR is a major function of SAA. This is because it allows cholesterol to remain in damaged tissues where it is needed for repair and regeneration of membranes and to transport cholesterol and clear lipid debris from bacteria and damaged areas of tissue (Eriksen *et al.*, 1993; Landman, 1998). During an APR, SAA prevents oxidative tissue damage and can recruit immune cells to localised areas of inflammation (Uhlar and Whitehead, 1999). SAA is immunomodulatory, inhibiting pyrexia and down regulating pro-inflammatory events during an APR (Shainkin-Kestenbaum *et al.*, 1991; Uhlar and Whitehead, 1999). In both mammals and birds, persistently elevated serum SAA levels, together with ongoing inflammatory and/or infectious disease may result in the formation of AA amyloid protein. The accumulation of this proteinaceous SAA derivative in organs and tissues gives rise to the disease AA amyloidosis (Röcken and Shakespeare, 2002).

AA amyloidosis in chickens and other Galliform species was rare until Landman *et al.* (1994) reported a new syndrome of avian amyloidosis in a flock of heavy breed, brown layer hens. Birds showed signs consistent with chronic arthritis, with inflammatory lesions in the joints and tendon sheaths. These lesions were found to be amyloid giving rise to a new arthropathic classification of amyloidosis in birds, of which Galliformes are predisposed (Landman, 1998). Serum amyloid A, when investigated in amyloidotic birds was found to be significantly higher than in healthy controls (Alasonyalilar *et al.,* 2006).

Nazifi *et al.* (2010; 2011) found significant increases in SAA in chickens infected with infectious bursal disease virus (IBDV) and infectious bronchitis (IB) virus. These studies show only mild, one and a half to two fold increases in plasma SAA concentration in response to the viral infections. Earlier work by Upragarin (2005) found SAA increased as a result of turpentine and *Staphylococcus aureus* injection with SAA levels increasing from undetectable levels pre-injection to increases of 100-1000 fold 12 hours post injection. At 72 hours post injection the mean SAA concentration of injected birds was 77.23 mg/L. Alasonyalilar *et al.* (2006) found severely amyloidotic birds to have mean serum SAA concentration of 42.53 mg/l. However, in this latter study the control birds had serum concentrations of 8.48 mg/l, much higher than control levels reported in Nazifi *et al.* (2010; 2011) and Upragarin (2005). The study by Upragarin (2005) is the only one to use an ELISA utilising specific chicken SAA antibody, the others use a Murine antibody. Further work is required to develop a chicken specific antibody assay to allow further study to determine the effects of bacterial and viral infections of SAA concentrations in chickens.

A1-ACID GLYCOPROTEIN

α1-acid glycoprotein (AGP) is a highly glycosylated protein synthesised and secreted by hepatocytes (Murata *et al.,* 2004; Ceron *et al.*, 2005). Like albumin, α1-acid glycoprotein is an important binding protein in plasma and a natural anti-inflammatory agent. It may help with the clearance of lipopolysaccharide (LPS), by binding directly with LPS and neutralising its toxicity (Murata *et al.,* 2004). α1-acid glycoprotein, a moderate APP in most mammals, appears to have a significant role in the early stages of inflammation and infection in chickens.

A number of studies have investigated AGP in chickens in response to both bacterial and viral infection. Following a single *Escherichia coli* LPS injection AGP plasma concentrations increased significantly 12 hours post injection with serum levels increasing four-fold to peak 24 hours post injection (Takahashi *et al.*, 1998). Nakamura *et al.* (1998) found infections with *E. coli* LPS resulted in an increase in AGP that peaked 48 hours post infection with a return to normal levels over 7 to 14 days. Repeated injections of *E. coli* LPS was found to lower the responses of plasma AGP concentrations (Takahashi *et al.,* 1995) while repeated injections of LPS at different concentrations resulted in different AGP profiles, with the higher concentration of LPS giving higher AGP values (Takahashi *et al.,* 1998). Age and sex do not significantly alter AGP concentrations (Takahashi *et al.,* 1995). Adler *et al.* (2001), using *Salmonella typhimurium* LPS and turpentine to illicit an APR found AGP levels increased four-fold, peaking 24 hours post infection before returning to normal levels six days post infection.

A number of studies have investigated AGP during viral infections both with and without vaccination. Experimental infection with infectious bronchitis (IB) and infectious larygotracheitis virus (ILTV) increased AGP serum concentrations, with AGP higher in the IB than the ILTV infected birds (Nakamura *et al.,* 1996). Inoue *et al.* (1997) found significant increases in serum AGP as a result of infection with a highly virulent

infectious bursal disease virus (IBDV) strain and a virulent IBDV reference strain. The highly virulent strain increased AGP concentrations six fold and the virulent reference strain three fold. The chickens infected with the highly virulent strains also had extensive lesions in addition to the severe bursal lesions. Chickens receiving the attenuated vaccine strain of IBDV showed only a slight increase in AGP and did not develop any lesions in the bursa or liver (Inoue *et al.,* 1997). Chickens challenged with low pathogenic avian influenza (LPAI) showed increases in AGP up to 48 post infection; however those that received LPAI vaccine were shown to have significantly lower AGP concentrations than unvaccinated controls (Sylte and Suarez, 2012).

AGP is a highly glycosylated APP, and several AGP glycoforms may be present normally in plasma. The glycosylation degree of AGP may be dramatically modified during disease (Ceciliani and Pocacqua, 2007). To study the glycan composition of AGP lectins, proteins that bind to specific carbohydrate groups can be used. The degree of branching of AGP can be studied by means of the interaction with the lectin concanavalin A (con A), that binds to α-linked mannose residues, two mannose molecules being required to interact with the lectin (Ceciliani and Pocacqua, 2007). Inoue *et al.* (1997) investigated the reactivity of AGP to con A in chickens experimentally infected with infectious bursal disease virus (IBDV). In control chickens of the total AGP, 47% was con A un-reactive and 63% con A reactive AGP. Inoculating birds with the highly virulent IBVD strain, saw con A reactive AGP increase to 80% of total AGP in the early stages of infection and infection with LPS increased con A to 87% of the total AGP (Inoue, *et al.*, 1997). The biological effects of AGP are dependent upon its glycosylation, with con-A un-reactive AGP exhibiting a stronger inhibitory effect of lymphocyte proliferation than the Con-A reactive. In addition, the inhibitory effects of AGP on neutrophil superoxide anion generation depends on the glycan structure with con A un-reactive being less inhibitory that the con A reactive (Inoue *et al.*, 1997). Although very little research has been undertaken on the glycosylation of chicken AGP it is clear from this work that the glycosylation pattern was modified as a result of IBDV.

α1-acid glycoprotein is one of the most widely measured APPs in chickens owing to the commercial availability of easy to use and chicken specific AGP kits (ECOS Institute, Miayagi, Japan). As a result AGP measurement has been used in a wide variety of poultry research areas most notably in the area of nutrition where experimental diets are followed by an immunogen challenge usually in the form of a bacterial LPS and the AGP concentration determined. The changes in AGP concentrations as a result of nutritional changes have been particularly well documented by Takahashi *et al.* (1995; 2002; 2009) (*Table 2*)

The use of APPs as a physiological marker for overall health and welfare is another potential use of APP measurement. In comparing different egg laying systems Salamano *et al.* (2010) compared the serum concentrations of AGP and albumin. Mean AGP serum concentrations, when measured at two weeks and two months post arrival at accommodation, were higher in hens kept in conventional and modified cages than hens in a free range system. However after four months the free range hens had higher mean AGP than both modified and conventionally caged groups, with the mean AGP of the free range hens having increased significantly from two months previously. AGP was used to evaluate stress and immunity of organically produced broilers compared to traditionally produced broilers. The organic broilers had serum AGP concentrations that were significantly higher ($P=<0.001$) than the conventionally produced broilers, this was attributed to restriction of prophylactic medicines coupled with access to the outside (Tuyttens *et al.,* 2008)..

Table 2 Studies of bacterial, viral, parasitic, immune-mediated and metabolic diseases in chickens that have measured acute phase proteins.

Infectious agent		APP	Reference
Viral	Infectious bursal disease virus	CP	Nazifi *et al.*, 2010
		SAA	Nazifi *et al.*, 2010
		PIT54	Nazifi *et al.*, 2010
		FB	Nazifi *et al.*, 2010
		OVT	Xie *et al.*, 2002b
		MBL	Nielsen *et al.*, 1998a; 1999
	Infectious bronchitis virus	SAA	Nazifi *et al.*, 2011
		OVT	Xie *et al.*, 2002b
		AGP	Nakamura *et al.*, 1996
		MBL	Juul-Madsen *et al.*, 2003; 2007
	Respiratory enteric orphan virus	OVT	Xie *et al.*, 2002b
	Fowl Poxvirus	OVT	Xie *et al.*, 2002b
	Infectious laryngotracheitis virus	OVT	Xie *et al.*, 2002b
		AGP	Nakamura *et al.*, 1996
		MBL	Nielsen *et al.*, 1998b; 1999
	Low pathogenic avian influenza	AGP	Sylte and Suarez, 2012
		OVT	Sylte and Suarez, 2012
Bacterial	*Escherichia coli* (LPS)	CP	Butler *et al.*, 1972
			Takahashi *et al.*, 2009
		OVT	Xie *et al.*, 2002b
			Hallquist and Klasing, 1994
		AGP	Takahashi *et al.*, 1994; 1995; 2009
			Nakamura *et al.*, 1998
			Buyse *et al.*, 2007
		HX	Barnes *et al.*, 2002
			Buyse *et al.*, 2007
		CRP	Patterson and Mora 1964; 1965
	Salmonella ser Typhimurium (LPS)	CP	Song *et al.*, 2009
			Koh *et al.*, 1996
		OVT	Xie *et al.*, 2000
		HX	Adler *et al.*, 2001
		PIT54	Millet *et al.*, 2007
		MBL	Millet *et al.*, 2007
	Salmonella ser Gallinarum	Cp	Garcia *et al.*, 2009
		PIT54	Garcia *et al.*, 2009
		OVT	Garcia *et al.*, 2009
		HX	Garcia *et al.*, 2009
	Staphylococcus aureus	SAA	Chamanza *et al.*, 1999a
			Upragarin, 2005
		CRP	Patterson and Mora, 1964
	Pasteurella multocida	MBL	Schou *et al.*, 2010
		CRP	Patterson and Mora, 1964
Parasitic	Eimeria tenella	CP	Georgieva *et al.*, 2010
		PIT54	Georgieva *et al.*, 2010
		FB	Georgieva *et al.*, 2010
		OVT	Rath *et al.*, 2009
		CP	Richards and Augustine, 1988
	Eimeria maxima	OVT	Rath *et al.*, 2009
	Eimeria acervulina	CP	Richards and Augustine, 1988
	Histomoniasis	CRP	Patterson and Mora, 1964
Immune mediated	Autoimmune vitiligo	OVT	Rath *et al.*, 2009
Metabolic	Tibial dyschondroplasia	OVT	Rath *et al.*, 2009
	Pulmonary hypertension	OVT	Rath *et al.*, 2009
	Femoral head separation	OVT	Durairaj *et al.*, 2009

OVOTRANSFERRIN

The transferrins are a family of metal binding transport proteins with an *in vivo* preference for binding ferric iron (Aguilera *et al.*, 2003). Mammals have serum transferrin, synthesised in the liver and lactoferrin a major milk protein, synthesised in the mammary gland. Chickens have neither of these proteins, instead synthesising ovotransferrin (OVT) in the liver in response to proinflammatory cytokines and in the oviduct under the influence of oestrogen (Hallquist and Klasing, 1994). Historically the word conalbumin was used to describe both the egg and serum transferrin in chickens.

Williams (1968) as well as advocating ovotransferrin (OVT) as a more informative name to describe the egg protein, demonstrated that both serum and egg OVT were the same protein differing only in their glycosylation. More recently serum transferrin has adopted the 'ovo-' and the word ovotransferrin is frequently used to describe both egg and serum transferrin in chickens. OVT is a positive APP in chickens (Hallquist and Klasing, 1994; Xie *et al.*, 2002a) with a number of studies confirming that serum OVT increases during an APR due to a variety of experimentally induced infections and inflammatory processes (*Table 2*). This differs from mammals where serum transferrin is a negative APP with expression being down regulated during inflammation (Ceron *et al.*, 2005). Egg OVT responds in a negative fashion during an APR, with the oviduct magnum decreasing mRNA synthesis for egg OVT with a resultant cessation to egg laying (Hallquist and Klasing, 1994).

Serum OVT like other members of the transferrin family is an antibacterial protein, able to sequester, store and transport iron. Sequestering iron deprives bacteria of the iron essential for growth (Xie *et al.*, 2002b). A direct action on the Gram negative bacteria *E. coli* has been identified as OVT is able to permeate the outer membrane causing bacteriostasis (Aguilera *et al.,* 2003). OVT has immunomodulatory effects inducing respiratory burst activity and degranulation in heterophils and macrophages, and facilitates tissue remodelling and angiogenesis, aiding post inflammatory repair of tissues (Xie *et al.,* 2002c). Xie *et al.* (2002b) investigated the serum OVT levels in response to a number of viruses and *E. coli* (*Table 3*). Rath *et al.* (2009) compared changes in the serum OVT concentration due to infectious diseases, metabolic diseases and autoimmune disease. Infectious diseases showed the biggest elevation, increasing OVT up to four fold. Autoimmune vitiligo resulted in an increase in serum OVT though the birds with tibial dyschondroplasia, pulmonary hypertension syndrome and femoral head separation disorder did not show elevations in serum OVT (Durairaj *et al.,* 2009; Rath *et al.*, 2009). It was postulated from these results that as well as inflammation or infection, tissue injury was needed to evoke a rise in OVT (Rath *et al.*, 2009). It appears that increases in OVT are only evident with pathogenic challenge and the increases seen in birds with the autoimmune disorder was most likely due to the pathologically high levels of proinflammatory cytokines that are stimulatory to OVT synthesis. In investigating the response of APPs to LPAI vaccination and infection, Sylte and Suarez (2012) also measured OVT alongside AGP and found that chickens receiving a LPAI vaccine had significantly lower serum OVT at 48 and 96 hours post infection than those chickens that did not receive the vaccine.

CERULOPLASMIN

Ceruloplasmin (CP) is a vertebrate plasma protein and contains over 95% of copper found in the plasma (Martínez-Subiela *et al.,* 2007). Avian blood is very low in copper compared to mammals and has lower serum levels of CP (Disilvestro and Harris, 1985). Ceruloplasmin is a multifunctional protein both storing and transporting copper within the body. Ceruloplasmin donates copper in a regulatory fashion with evidence of CP receptors on the membranes of numerous different cell types, supporting CP's role as a

cell signalling multifunctional enzyme within many different metabolic pathways (Floris *et al.,* 2000). Ceruloplasmin is an antioxidant protein, scavenging reactive oxygen species and preventing their formation through its involvement in iron homeostasis. Historically, preventing iron-induced oxidative stress has been considered to be the principle role of the protein *in vivo* (Floris *et al.,* 2000).

Butler *et al.* (1972) first described CP as an APP in chickens when it was noted that a single injection of endotoxin from *E. coli* increased serum CP levels by two to five fold, the increase varying with the strain of *E. coli* used. The maximal increase in CP levels was 24 hours post injection with levels still detectable 48 hours after injection. Further studies confirmed CP as a positive acute phase reactant. *Salmonella gallinarum* delivered into the crop of chickens showed CP to increase the quickest of all the APPs measured in this study, with peak serum CP levels measured three to five days post infection (Garcia *et al.,* 2009). Serum CP will increase significantly in response to *Eimeria tenella* infection (Richards and Augustine, 1988; Georgieva *et al.,* 2010) and combined *E. coli* and *E. tenella* infection (Georgieva *et al.,* 2010). Infection of *Eimeria acervulina* did not significantly increase CP concentrations (Richards and Augustine, 1988). Nazifi *et al.* (2010) found CP to decrease in chickens infected IBDV and the decrease was even more pronounced in the most severely infected. It would be valuable to establish whether CP shows a negative APP response in other viral infections in chickens or if this is an IBDV associated response.

FIBRINOGEN

Fibrinogen (FB) is a soluble glycoprotein present in the plasma of all vertebrates (Ceron *et al.,* 2005). Synthesised by hepatocytes, FB functions as a key regulator of inflammation during disease. The pro-inflammatory functions of FB, fibrin and derivative peptides are associated with their ability to bind to and activate a wide range of immune cells. Importantly the pro-inflammatory functions of FB are a product of FB signalling through binding sites that do not overlap with those involved in the coagulation cascade (Davalos and Akassoglou, 2012). During an APR the vascular disruption associated with pathological events such as inflammation, infection and tissue injury cause the concentration of fibrinogen in the blood to increase (Davalos and Akassoglou, 2012). As well as providing a substrate for fibrin formation FB also provides a matrix for the migration of inflammatory related cells and for tissue repair (Murata *et al.,* 2004).

FB is an established APP in chickens, increasing two to two and a half fold as a result of turpentine injection, with levels peaking two to three days post injection and returning to normal values by seven days (Amrani *et al.,* 1986; Grieninger *et al.,* 1986). Georgieva *et al.* (2010) found FB levels increased significantly ($P=<0.001$) in response to combined infection of *E. coli* and *E. tenella* and with a single infection of *E. coli* or *E. tenella*, with serum concentrations increasing fourfold with the *E. coli* infection. FB when studied during IBDV infection was not shown to increase significantly (Nazifi *et al.,* 2010).

FIBRONECTIN

Fibronectin (FN) is large a molecular weight glycoprotein synthesised in hepatocytes as a soluble plasma FN and in tissues as an insoluble structural form of FN, synthesised in fibroblasts, epithelial cells and other differentiated cell types (Labat-Robert, 2012). FN is widely expressed in multiple cell types and is critically important in vertebrate development (Pankov and Yamada, 2002). In tissues FN is an extracellular matrix (ECM) glycoprotein playing an important role in the assembly and organisation of the matrix and mediating a wide variety of cellular interactions. Within the matrix FN has a wide variety of functional activities including cell adhesion, migration, growth and differentiation. FN is able to bind biologically important molecules having binding

sites for bacteria, collagen, fibrin, FN itself and heparin (Pankov and Yamada, 2002; Labat-Robert, 2012). Plasma FN is a minor APP, increasing during an APR and able to diffuse into tissues and form part of the fibrillar matrix (Labat-Robert, 2012) where, together with tissue FN, it plays an important and active role in wound healing.

In chickens, Amrani *et al.* (1986) found plasma FN levels increased two and a half fold as a result of turpentine injection, rapidly peaking two days post injection and remaining elevated 52 hours after the injection. To investigate the effect of increased temperature on FN concentration, broilers were exposed to an elevated temperature (42°C) for three hours; 28 hours after exposure FN concentration was significantly higher (JianHua *et al.*, 2000). In response to ACTH and dexamethasone administration, FN in chicken plasma increased 1.6 and 2.5 fold respectively from basal levels and remained elevated for 52 hours (LiCheng *et al.,* 2000).

Haem binding proteins

Under certain pathological conditions such as infection the red blood cell membrane can destabilise resulting in the lyses of the cell and leakage of haemoglobin into circulation (Wicher and Fries, 2006). Haemoglobin outside the cell is toxic, proinflammatory and pro-oxidative causing damage to cells and tissues and haem associated iron will support microbial growth (Ceron *et al.,* 2005; Wicher and Fries, 2010). Plasma proteins have therefore evolved that bind free haemoglobin and haem and mediate their removal from the circulation (Wicher and Fries, 2010).

PIT54

Haptoglobin is the major haemoglobin binding protein found in mammals. The haemoglobin binding protein in chickens was considered to be haptoglobin or a haptoglobin-like protein until detailed analysis of this protein in chickens and the chicken genome found there to be no gene coding a protein similar to mammalian haptoglobin. Instead the protein referred to as PIT54 was identified, which fulfils a similar role in chickens as haptoglobin does in mammals (Iwasaki *et al.,* 2001; Wicher and Fries, 2006). PIT54 is found only in avian species having been identified in geese, ostriches and emus. Although absent in chickens, haptoglobin is found in paleognathus birds (ostriches and emus) where haptoglobin has been conserved alongside PIT54 (Wicher and Fries, 2010).

Haptoglobin is an anti-inflammatory protein, it modulates prostaglandin synthesis and inhibits granulocyte chemotaxis and phagocytosis (Ceron *et al.,* 2005). PIT54 evolved from an anti-inflammatory protein which evolved haemoglobin binding ability (Wicher and Fries, 2010) and it is likely that PIT54, like haptoglobin retains these anti-inflammatory properties. PIT54 has been shown to have potent antioxidant properties, inhibiting superoxide production by phagocytes, thus inhibiting the overproduction of reactive oxygen species (Iwasaki *et al.,* 2001) that cause oxidative damage. PIT54 is antibacterial as binding free haemoglobin renders it unavailable for bacterial growth. Haemoglobin iron availability and the susceptibility to infection are intimately linked with pathological iron overload predisposing to bacterial infection (Ascenzi *et al.,* 2005).

Nazifi *et al.* (2010; 2011) found that chickens had no significant increase in PIT54 during IBDV infection, yet had a significant increase as a result of IB infection. As a result of parenteral administration of *S. typhimurium* LPS, mean PIT54 serum concentrations significantly increased 1.5 fold (Millet *et al.*, 2007). Garcia *et al.* (2009) found that intra-crop inoculation of *S. gallinarum* increased PIT54 throughout the course of a 10 day infection. Georgieva *et al.* (2010) compared serum PIT54 after

experimental infection with *E. coli, E. tenella* and combined infection of both pathogens. The increase in PIT54 was highly significant as a result of *E. coli* infection and combined infection however serum PIT54 levels did not increase significantly as a result of *E. tenella* infection alone.

HAEMOPEXIN

Haemopexin (HX) is a haem binding protein and an established APP in chickens (Grieninger *et al.*, 1986; Adler *et al.*, 2001). HX binds to the non-protein pyrrole rings within the haem molecule. Its predominant function is to sequester and transport haem, though it is a multifunctional protein having involvement in iron homeostasis, antioxidant production and signalling pathways that promote cell survival and gene expression (Tolosano *et al.,* 2010). Barnes *et al.* (2002), using *E. coli* LPS to induce an APR found a 2.6 fold increase in HX. Adler *et al.* (2001) used *S. typhimurium* LPS and complete Freund's adjuvant and found HX levels to increase three fold at 24 hours post infection, HX levels were still significantly higher than the control birds 14 days after the LPS challenge. Garcia *et al.* (2009) found HX to increase by approximately one and a half fold in response to intra crop *S. gallinarum*, with serum concentration decreasing from seven days post infection. Mammalian plasma HX declines between 24-72 hours post challenge. Establishing the behaviour of HX in response to different infections over a period longer than 14 days would allow HX behaviour during an APR to be fully characterised in chickens.

C-reactive protein

Originally named for its ability to bind C-polysaccharide of *Pneumococcus*, pneumonia, C-reactive protein (CRP) was the first described APP (Abernethy and Avery, 1941) and has been defined in humans as a highly sensitive marker of inflammation and tissue damage. CRP is a major APP in humans and dogs and is the most frequently measured APP in veterinary species (Eckersall and Bell, 2010). CRP binds directly to degenerating cells and cell remnants acts as an opsonin binding residues and polysaccharides on bacteria, fungi and parasites to activate complement and phagocytosis. CRP can also up regulate and down regulate cytokine production and chemotaxis (Gruys *et al.*, 2005; Cray *et al.*, 2009).

Patterson and Mora (1964) purified CRP from chicken serum and developed an assay to detect the presence or absence of CRP in chicken serum. They found CRP to be present in birds with *E. coli, Pasturella multocida* and *Staphylococcus aureus* infections, as well as histomoniasis and adjuvant injection. The authors also found CRP positive birds in a clinically normal population. These positive birds, on post mortem, had lesions consistent with chronic respiratory disease, highlighting the use of CRP as a potential biomarker for non-clinical disease. Whether CRP is a major APP in chickens as it is in humans and other veterinary species is yet to be established. Patterson and Mora (1965) found that CRP did not rise in chickens as quickly as it does in humans, whereby CRP was detectable 36-48 hours post infection in chickens, compared to 16 -18 hours in humans. It was also noted that the CRP concentration, though not quantified, appeared lower in chickens than in comparative human samples. A more recent study investigated CRP serum concentrations using a human CRP kit and found CRP concentrations to increase (Sohail *et al.,* 2010).

Mannan binding lectin

Mannan binding lectin (MBL) belongs to a group of proteins called collectins which have a carbohydrate recognition domain able to bind a variety of microorganisms including bacteria, viruses, fungi and parasites. Mannan binding lectin, upon recognition of infectious agents, activates a number of cellular defence mechanisms including phagocytosis, modulation of cytokines and immunoglobulin secretion. Mannan binding lectin is also able to activate the complement system through a distinct MBL pathway (Nielsen *et al.*, 1999; Juul-Madsen *et al.*, 2003; Schou *et al.*, 2010).

In chickens, MBL behaves as an minor APP with ILTV, IBDV and IB, increasing serum MBL two fold with peak increases seen three to seven days post infection, with a return to normal values at six to ten days (Nielsen *et al.*, 1998a; Nielsen *et al.*, 1999). In relation to bacterial infections, MBL showed a significant two fold increase when chickens were inoculated with *S. typhimurium* LPS (Millet *et al.*, 2007). Although classed as a minor acute phase reactant, MBL plays an active and prominent role in the immune response. When examining the baseline serum MBL concentrations in relation to intra-tracheal inoculation of *P. multocida,* Schou *et al.* (2010) found that chickens had significantly higher mean MBL concentrations prior to infection compared to four weeks post infection, and those birds that developed a systemic infection (as determined by *P. multocida* invasion of spleen) following inoculation, had lower mean baseline MBL than those with no splenic invasion, suggesting MBL plays a role in the chickens immune system in protecting against systemic *P. multocida* infection. Immuno-histochemical staining for MBL in healthy chickens detected MBL in the cytoplasm of a few hepatocytes and germinal centres of the caecal tonsils. In birds infected with ILTV an intense staining for MDL was detected in the cytoplasm of all hepatocytes and on the surface of and inside ILTV infected cells. IBDV infected birds had intense staining for MBL in the hepatocyte cytoplasm but no staining was observed in the follicles of the bursa of Fabricius, though MBL was present in the spleen. The presence of MBL in infected and lymphatic tissues is indicative of an important and active role within the immune system (Nielsen *et al.*, 1998b). Studies have also revealed expression of MBL to be genetically influenced. Juul-Madsen *et al.* (2007) selected two lines of chickens for low and high levels of serum MBL concentration. At the sixth generation, MBL levels were found to be significantly higher in the high line and lower in the low line. Furthermore, when experimentally infected with IB virus,. there was a 2.3 fold and 1.6 fold increase in MBL in the low and high lines respectively (Juul-Madsen *et al.,* 2007).

Measuring APPs in chickens

APPs can be measured in plasma or serum. However, in avian species, plasma is more often used because it is easier to collect than serum and yields a larger sample volume (Hrubec *et al.*, 2002). Blood samples can be collected into anticoagulants such as EDTA or heparin and then be centrifuged to separate the cells. Heparin is reported as the preferable anticoagulant for avian species owing to its low biologic activity and lack of interference with analyte detection (Hrubec *et al.*, 2002). Although the effect of both anticoagulants on plasma APP measurement has not been investigated in chickens, in mammalian species CP values were significantly higher with heparin than EDTA and increases in haptoglobin concentrations have been found when measured in a heparin sample (Ceron *et al.*, 2005). The effect of anticoagulant choice on APP concentrations appears to be small in mammalian species (Ceron *et al.*, 2005) however determining the

effect of anticoagulants on measurable APP concentrations in chickens would allow more meaningful comparison of different studies, especially for the minor APPs, where only two or three fold changes are detected. If measuring fibrinogen or fibronectin (which co-precipitates with fibrinogen, (Labat-Robert and Robert 2012) then a plasma sample is essential as a serum sample will not contain the clotting constituents.

Samples should not be haemolysed or lipaemic as this will affect the result of APPs measured by immunoturbidimetric assays (Ceron *et al.*, 2005) Samples can be frozen and stored at -20°C and APPs remain stable at this temperature for up to two months (Ceron *et al.*, 2005). Lower temperatures are required for longer storage. Freeze thaw cycles should be avoided, and samples should be alliquoted if necessary.

Table 3 contains references for methods or commercial kits that have been used to measure chicken APPs. All methods used should be validated for measuring chicken APPs especially when using antibodies against mammalian APPs. The availability of the AGP kit, which utilises chicken specific antibody, makes this one of the most widely measured APPs in chickens. Many labs investigating chicken APPs develop their own assays by raising antibody to the purified chicken APP.

Table 3 How to measure acute phase proteins in chickens.

APP	Measurement
Ceruloplasmin	Indirectly measured using p-phenylenediamine (PPD) oxidase activity (Sunderman and Nomoto 1970; Martínez-Subiela *et al.*, 2007)
Fibrinogen	Heat precipitation method (Thrall *et al.*, 2004)
Fibronectin	ELISA (Lynagh *et al.*, 2000)
OVT	ELISA (Xie *et al.*, 2002b; Rath *et al.*, 2009)
PIT54	Commercially available kits for measuring haptoglobin are based on the haemoglobin binding activity (Eckersall *et al.*, 1999) are effective with chicken PIT54
Haemopexin	Rocket gel electrophoresis using a rabbit anti-chicken hemopexin (Adler *et al.*, 2001)
SAA	Mouse SAA antibody solid phase sandwich ELISA available from Tridelta (Ireland) (Nazifi *et al.*, 2010)
MBL	Sandwich ELISA (Laursen *et al.*, 1998)
α -1- glycoprotein	Specific chicken single immunodiffusion test kit available using anti-chicken AGP antibodies of rabbit origin (ECOS Institute, Miyagi, Japan)

As well as ELISA methods, gel based methods can also be used to quantify APPs. Garcia *et al.* (2009) used SDS PAGE gels with samples compared to purified APPs and the concentration of the APP calculated using a scanning densitometer. Combined antibody and gel methods also work very well, such as the AGP kit (*Table 3*) and that used by (Adler *et al.*, 2001) to measure HX. Commercially available kits for measuring haptoglobin in mammals also respond to PIT54 in chickens. APPs have been used in a number of studies of infectious diseases. *Table 2* summarises the APPs that have been measured in chickens with bacterial, viral, parasitic, immune-mediated and metabolic diseases.

Conclusions

Chamanza *et al.* (1999b) produced a substantial review of APPs in the domestic fowl in 1999. At the time measuring APPs in poultry species was in its infancy, no kits were commercially available and the number of diseases in which APPs had been studied was

limited. Since then research into APPs in poultry species, especially the chicken, has developed widely to include many infectious diseases as well as welfare and nutritional studies. Since the last review many APPs have been identified in the chicken and more detailed information about chicken APPs, their structure and how they respond during an APR is available.

Many of the APPs still require further research either to fully determine their behaviour in the chicken or to pave way for the development of chicken specific assays which would expand the measurement of APPs in chickens beyond the research environments. A small number of proteomic investigations in chickens, have harnessed two-dimensional protein methodologies and found them to yield a high level of information on how the plasma proteome changes in response to disease. This expanding area may give rise to the identification of other APPs and biomarkers of disease. Other production species have utilised APP measurement as biomarkers for health and disease and as a selection tool for breeding programmes. Ongoing work is continuing to elaborate on the role APPs play in the immune system of the chicken and this will be advanced greatly by the increased availability of assays validated for chickens and concurrent proteomic investigations.

References

ABERNETHY, T. and AVERY, O. (1941) The occurrence during acute infections of a protein not normally present in the blood. *The Journal of Experimental Medicine* **73**: 173-182.

ADLER, K.L., PENG, P.H., PENG, R.K. and KLASING, K.C. (2001) The Kinetics of Hemopexin and α1-Acid Glycoprotein Levels Induced by Injection of Inflammatory Agents in Chickens Inflammatory Agents in Chickens. *Avian Diseases* **45**: 289-296.

AGUILERA, O., QUIROS, L.M. and FIERRO, J.F. (2003) Transferrins selectively cause ion efflux through bacterial and artificial membranes. *FEBS Letters* **548**: 5-10.

ALASONYALILAR, A., SEVIMLI, A., MISIRLIOGLU, D. and UGUZ, C. (2006) Chronic amyloid arthropathy and increased serum amyloid levels in brown layers. *The Bulletin of the Veterinary Institute in Pulawy* **50**: 557-560.

AMRANI, D.L., MAUZY-MELITZ, D. and MOSESSON, M.W. (1986) Effect of hepatocyte-stimulating factor and glucocorticoids on plasma fibronectin levels. *The Biochemical Journal* **238**: 365-371.

ASCENZI, P., BOCEDI, A., VISCA, P., ALTRUDA, F., TOLOSANO, E., BERINGHELLI, T. and FASANO, M. (2005) Hemoglobin and heme scavenging. *IUBMB life* **57**: 749-759.

BARNES, D.M., SONG, Z., KLASING, K.C. and BOTTJE, W. (2002) Protein metabolism during an acute phase response in chickens. *Amino acids* **22**: 15-26.

BUTLER, E.J., CURTIS, M.J., HARRY, E.G. and DEB, J.R. (1972) Effects of Escherichia coli endotoxins on plasma para-phenylenediamine oxidase (caeruloplasmin) activity in the domestic fowl. *Journal of Comparative Pathology* **82**: 299-306.

BUYSE, J., SWENNEN, Q., NIEWOLD, T.A., KLASING, K.C., JANSSENS, G.P.J., BAUMGARTNER, M. and GODDEERIS, B. (2007) Dietary L-carnitine supplementation enhances the lipopolysaccharide-induced acute phase protein response in broiler chickens. *Veterinary Immunology and Immunopathology* **118**: 154-159.

CECILIANI, F. and POCACQUA, V. (2007) The acute phase protein 1-acid glycoprotein: a model for altered glycosylation during diseases. *Current Protein and Peptide Science* **8**: 91-108.

CERON, J.J., ECKERSALL, P.D. and MARTÝNEZ-SUBIELA, S. (2005) Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Veterinary Clinical Pathology* **34**: 85-99.

CHAMANZA, R., TOUSSAINT, M.J.M., VAN EDEREN, A.M., VAN VEEN, L., HULSKAMP-KOCH, C. and FABRI, T.H.F. (1999a) Serum amyloid a and transferrin in chicken. A preliminary investigation of using acute-phase variables to assess diseases in chickens. *Veterinary Quarterly* **21**: 37-41.

CHAMANZA, R., VAN VEEN, L., TIVAPASI, M.T. and TOUSSAINT, M.J.M. (1999b) Acute phase proteins in the domestic fowl. *World's Poultry Science Journal* **55**: 61-71.

CRAY, C., ZAIAS, J. and ALTMAN, N.H. (2009) Acute phase response in animals: a review. *Comparative Medicine* **59**: 517-526.

DAVALOS, D. and AKASSOGLOU, K. (2012) Fibrinogen as a key regulator of inflammation in disease. *Seminars in Immunopathology* **34**: 43-62.

DISILVESTRO, R.A. and HARRIS, E.D. (1985) Purification and partial characterization of ceruloplasmin from chicken serum. *Archives of Biochemistry and Biophysics* **241**: 438-446.

DURAIRAJ, V., OKIMOTO, R., RASAPUTRA, K., CLARK, F.D. and RATH, N.C. (2009) Histopathology and serum clinical chemistry evaluation of broilers with femoral head separation disorder. *Avian Diseases* **53**: 21-25.

ECKERSALL, P.D., DUTHIE, S., SAFI, S., MOFFATT, D., HORADAGODA, N.U., DOYLE, S., PARTON, R., BENNETT, D. and FITZPATRICK, J.L. (1999) An Automated Biochemical Assay for Haptoglobin: Prevention of Interference from Albumin. *Comparative Haemotology International* **9**: 117-124.

ECKERSALL, P.D. and BELL, R. (2010) Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. *The Veterinary journal* **185**: 23-27.

ERIKSEN, N., MEEK, R.L. and BENDITT, E.P. (1993) The SAA lipoprotein family, in: MACKIEWICZ, A., KUSHNER, I. & BAUMANN, H. (Eds) *Acute Phase Proteins: Molecular biology, Biochemistry and Clinical Applications*, pp. 93-106 (CRC Press, Boca Raton, Florida).

FLORIS, G., MEDDA, R., PADIGLIA, A. and MUSCI, G. (2000) The physiopathological significance of ceruloplasmin. *Biochemical Pharmacology* **60**: 1735-1741.

GARCIA, K.O., BERCHIERI-JUNIOR, A., SANTANA, A.M., FREITAS-NETO, O.C. and FAGLIARI, J.J. (2009) Experimental infection of commercial layers using a Salmonella enterica serovar Gallinarum strain: Leukogram and serum acute-phase protein concentrations. *Brazillian Journal of Poultry Science* **11**: 263-270.

GEORGIEVA, T.M., KOINARSKI, V.N., URUMOVA, V.S., MARUTSOV, P.D., CHRISTOV, T.T., NIKOLOV, J., CHAPRAZOV, T., WALSHE, K., KAROV, R.S., GEORGIEV, I.P. and KOINARSKI, Z.V. (2010) Effects of Escherichia coli infection and Eimeria tenella invasion on blood concentrations of some positive acute phase proteins (haptoglobin (PIT 54), fibrinogen and ceruloplasmin). *Revue de Medecine Veterinaire* **161**: 84-89.

GRIENINGER, G., LIANG, T.J., BEUVING, G., GOLDFARB, V., METCALFE, S.A. and MULLER-EBERHARD, U. (1986) Hemopexin is a developmentally regulated, acute-phase plasma protein in the chicken. *The Journal of Biological Chemistry* **261**: 15719-15724.

GRUYS, E., TOUSSAINT, M.J.M., NIEWOLD, T.A. and KOOPMANS, S.J. (2005) Acute phase reaction and acute phase proteins. *Journal of Zhejiang University. Science B* **6**: 1045-1056.

HALLQUIST, N.A. and KLASING, K.C. (1994) Serotransferrin, ovotransferrin and metallothionein levels during an immune response in chickens. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **108**: 375-384.

HRUBEC, T., WHICHARD, J.M., LARSEN, C.T. and PIERSON, M.S. (2002) Plasma versus serum: specific differences in biochemical analyte values. *Journal of Avian Medicine and Surgery* **16**: 101-105.

INOUE, M., SATOH, W. and MURAKAMI, H. (1997) Plasma alpha 1-acid glycoprotein in chickens infected with infectious bursal disease virus. *Avian Diseases* **41**: 164-170.

IWASAKI, K., MORIMATSU, M., INANAMI, O., UCHIDA, E., SYUTO, B., KUWABARA, M. and NIIYAMA, M. (2001) Isolation, characterization, and cDNA cloning of chicken turpentine-induced protein, a new member of the scavenger receptor cysteine-rich (SRCR) family of proteins. *The Journal of Biological Chemistry* **276**: 9400-9405.

JIANHUA, L., LICHENG, L., JIUSHAN, J. and HONGKUI, L. (2000) Effects of high temperature on plasma fibronectin levels and serum biochemical parameters in broilers. *Chinese Journal of Veterinary Science* **20**: 591-593.

JUUL-MADSEN, H.R., MUNCH, M., HANDBERG, K.J., SØRENSEN, P., JOHNSON, A.A., NORUP, L. R. and JØRGENSEN, P.H. (2003) Serum levels of mannan-binding lectin in chickens prior to and during experimental infection with avian infectious bronchitis virus. *Poultry Science* **82**: 235-241.

JUUL-MADSEN, H.R., NORUP, L.R., HANDBERG, K.J. and JØRGENSEN, P.H. (2007) Mannan-binding lectin (MBL) serum concentration in relation to propagation of infectious bronchitis virus (IBV) in chickens. *Viral Immunology* **20**: 562-570.

KOH, T.S., PENG, R.K. and KLASING, K.C. (1996) Dietary copper level affects copper metabolism during lipopolysaccharide-induced immunological stress in chicks. *Poultry Science* **75**: 867-872.

LABAT-ROBERT, J. (2012) Cell-Matrix interactions, the role of fibronectin and integrins. A survey *Pathologie-Biologie* **60**: 15-19.

LABAT-ROBERT, J. and ROBERT, L. (2012) Fifty years of structural glycoproteins. *Pathologie-Biologie* **60**: 66-75.

LANDMAN, W., GRUYS, E. and DWARS, R. (1994) A syndrome associated with growth depression and amyloid arthropathy in layers: a preliminary report. *Avian Pathology* **23**: 461- 470.

LANDMAN, W.J.M. (1998) Amyloid Arthropathy. *Ph. D. thesis*, Utrecht University.

LAURSEN, S.B., HEDEMAND, J.E., NIELSEN, O.L., THIEL, S., KOCH, C. and JENSENIUS, J.C. (1998) Serum levels, ontogeny and heritability of chicken mannan-binding lectin (MBL). *Immunology* **94**: 587-593.

LAURSEN, S.B. and NIELSEN, O.L. (2000) Mannan-binding lectin (MBL) in chickens: molecular and functional aspects. *Developmental and Comparative Immunology* **24**: 85-101.

LICHENG, L., JIANHUA, L., JIUSHAN, J., JIXUN, Z. and CHENG, H. (2000) Effect of ACTH and dexamethasone on plasma fibronectin levels and serum biochemical parameters in broilers. *Journal of China Agricultural University* **5**: 106 -110.

LYNAGH, G.R., COLLINS, R.A. and KAISER, P. (2000) Development and use of monoclonal antibodies to chicken fibronectin to show that the chicken hepatocellular carcinoma cell line, LMH, constitutively expresses fibronectin. *Research in Veterinary Science* **68**: 147-152.

MARTÍNEZ-SUBIELA, S., TECLES, F. and CERON, J.J. (2007) Comparison of two automated spectrophotometric methods for ceruloplasmin measurement in pigs. *Research in Veterinary Science* **83**: 12-19.

MILLET, S., BENNETT, J., LEE, K.A., HAU, M. and KLASING, K.C. (2007) Quantifying and comparing constitutive immunity across avian species. *Developmental and Comparative Immunology* **31**: 188-201.

MURATA, H., SHIMADA, N. and YOSHIOKA, M. (2004) Current research on acute phase proteins in veterinary diagnosis: an overview. *The Veterinary Journal* **168**: 28-40.

NAKAMURA, K., MITARAI, Y. and YOSHIOKA, M. (1998) Serum levels of interleukin-6, alpha1-acid glycoprotein, and corticosterone in two-week-old chickens inoculated with Escherichia coli lipopolysaccharide. *Poultry Science* **77**: 908-911.

NAKAMURA, K., IMAI, K. and TANIMURA, N. (1996) Comparison of the effects of infectious bronchitis and infectious laryngotracheitis on the chicken respiratory tract. *Journal of Comparative Pathology* **114**: 11-21.

NAZIFI, S., TABANDE, M.R., HOSSEINIAN, S.A., ANSARI-LARI, M. and SAFARI, H. (2011) Evaluation of sialic acid and acute-phase proteins (haptoglobin and serum amyloids A) in healthy and avian infection bronchitis virus-infected chicks. *Comparative Clinical Pathology* **20**: 69-73.

NAZIFI, S., DADRAS, H., HOSEINIAN, S.A., ANSARI-LARI, M. and MASOUDIAN, M. (2010) Measuring acute phase proteins (haptoglobin, ceruloplasmin, serum amyloid A, and fibrinogen) in healthy and infectious bursal disease virus-infected chicks. *Comparative Clinical Pathology* **19**: 283-286.

NIELSEN, O.L., SØRENSEN, P., HEDEMAND, J.E., LAURSEN, S.B. and JØRGENSEN, P.H. (1998a) Inflammatory response of different chicken lines and B haplotypes to infection with infectious bursal disease virus. *Avian Pathology* **27**: 181-189.

NIELSEN, O.L., JØRGENSEN, P.H., HEDEMAND, J., JENSENIUS, J.C., KOCH, C. and LAURSEN, S. B. (1998b) Immunohistochemical investigation of the tissue distribution of mannan-binding lectin in non-infected and virus-infected chickens *Immunology* **94**: 122-128.

NIELSEN, O.L., JENSENIUS, J.C., JØRGENSEN, P.H. and LAURSEN, S.B. (1999) Serum levels of chicken mannan-binding lectin (MBL) during virus infections; indication that chicken MBL is an acute phase reactant. *Veterinary Immunology and Immunopathology* **70**: 309-316.

OVELGÖNNE, J.H., LANDMAN, W.J., GRUYS, E., GIELKENS, A.L. and PEETERS, B.P. (2001) Identical amyloid precursor proteins in two breeds of chickens which differ in susceptibility to develop amyloid arthropathy. *Amyloid* **8**: 41-51.

PANKOV, R. and YAMADA, K.M. (2002) Fibronectin at a glance. *Journal of Cell Science* **115**: 3861-3863.

PATTERSON, L.T. and MORA, E.C. (1964) Occurance of a substance analogous to C-reactive protein in the blood of the domestic fowl. *Texas Reports on biology and medicine* **22**: 716-721.

PATTERSON, L.T. and MORA, E.C. (1965) The C-reactive protein response and disease resistance in domestic fowl. *Texas Reports on biology and medicine* **23**: 600-606.

PETERSEN, H.H., NIELSEN, J.P. and HEEDAARD, P.M.H. (2004) Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary Research* **35**: 163-187.

RATH, N.C., ANTHONY, N.B., KANNAN, L., HUFF, W.E., HUFF, G.R., CHAPMAN, H.D., ERF, G.F. and WAKENELL, P. (2009) Serum ovotransferrin as a biomarker of inflammatory diseases in chickens. *Poultry Science* **88**: 2069-2074.

RICHARDS, M.P. and AUGUSTINE, P.C. (1988) Serum and liver zinc, copper, and iron in chicks infected with Eimeria acervulina or Eimeria tenella. *Biological Trace Element Research* **17**: 207-219.

RÖCKEN, C. and SHAKESPEARE, A. (2002) Pathology, diagnosis and pathogenesis of AA amyloidosis. *Virchows Archiv* **440**: 111-122.

SALAMANO, G., MELLIA, E., TARANTOLA, M., GENNERO, M.S., DOGLIONE, L. and SCHIAVONE, A. (2010) Acute phase proteins and heterophil:lymphocyte ratio in laying hens in different housing systems. *The Veterinary Record* **167**: 749-751.

SCHOU, T.W., PERMIN, A., CHRISTENSEN, J.P., CU, H.P. and JUUL-MADSEN, H.R. (2010) Mannan-binding lectin (MBL) in two chicken breeds and the correlation with experimental Pasteurella multocida infection. *Comparative Immunology, Microbiology and Infectious Diseases* **33**: 183-195.

SEVIMLI, A., MISIRLIOĞLU, D., POLAT, U., YALÇIN, M., AKKOÇ, A. and UĞUZ, C. (2005) The effects of vitamin A, pentoxyfylline and methylprednisolone on experimentally induced amyloid arthropathy in brown layer chicks. *Avian Pathology* **34**: 143-149.

SHAINKIN-KESTENBAUM, R., BERLYNE, G., ZIMLICHMAN, S., SORIN, H.R. NYSKA, M. and DANON, A. (1991) Acute phase protein, serum amyloid A, inhibits IL-1- and TNF-induced fever and hypothalamic PGE2 in mice. *Scandinavian Journal of Immunology* **34**: 179-183.

SOHAIL, M.U., IJAZ, A., YOUSAF, M.S., ASHRAF, K., ZANEB, H., ALEEM, M. and REHMAN, H. (2010) Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poultry Science* **89**: 1934-1938.

SONG, Z., ZHU, L., ZHAO, T., JIAO, H. and LIN, H. (2009) Effect of copper on plasma ceruloplasmin and antioxidant ability in broiler chickens challenged by lipopolysaccharide. *Asian-Australasian Journal of Animal Sciences* **22**: 1400-1406.

SUNDERMAN, F. and NOMOTO, S. (1970) Measurement of human serum ceruloplasminby its p-phenylenediamine oxidase activity. *Clinical Chemistry* **16**: 903-910.

SYLTE, M.J. and SUAREZ, D.L. (2012) Vaccination and acute phase mediator production in chickens challenged with low pathogenic avian influenza virus; novel markers for vaccine efficacy? *Vaccine* **30**: 3097-3105.

TAKAHASHI, K., AKIBA, Y., IWATA, T. and KASAI, M. (2002) Dietary conjugated linoleic acids alleviate early inflammatory response caused by lipopolysaccharide injection in male broiler chicks. *Animal Science Journal* **73**: 47-50.

TAKAHASHI, K., KAJI, N., AKIBA, Y. and TAMURA, K. (1994) Plasma alpha 1-acid glycoprotein concentration in broilers: influence of age, sex and injection of Escherichia coli lipopolysaccharide. *British Poultry Science* **35**: 427-432.

TAKAHASHI, K., MIYAKE, N. and OHTA, T. (1998) Changes in plasma alpha 1-acid glycoprotein concentration and selected immune response in broiler chickens injected with Escherichia coli lipopolysaccharide. *British Poultry Science* **39**: 152-155.

TAKAHASHI, K., TAKAGI, K. and AKIBA, Y. (2009) Effects of dietary glycine supplementation and fish meal on inflammatory responses in broiler chicks. *British Poultry Science* **50**: 479-486.

TAKAHASHI, K., YODOGAWA, S., AKIBA, Y. and TAMURA, K. (1995) Effect of dietary protein concentration on responses to Escherichia coli endotoxin in broiler chickens. *British Journal of Nutrition* **74**: 173-182.

THRALL, M.A., BAKER, D.C., CAMPBELL, T.W., DENICOLA, D.B., FETTMAN, M.J. and LASSEN, E.D. (2004) *Veterinary Hematology and Clinical Chemistry* (John Wiley & Sons).

TOLOSANO, E., FAGOONEE, S., MORELLO, N., VINCHI, F. and FIORITO, V. (2010) Heme scavenging and the other facets of hemopexin. *Antioxidants & Redox Signaling* **12**: 305-320.

TUYTTENS, F., HEYNDRICKX, M., DE BOECK, M., MOREELS, A., VAN NUFFEL, A., VAN POUCKE, E., VAN COILLIE, E., VAN DONGEN, S. and LENS, L. (2008) Broiler chicken health, welfare and fluctuating asymmetry in organic versus conventional production systems. *Livestock Science* **113**: 123-132.

UHLAR, C.M. and WHITEHEAD, A.S. (1999) Serum amyloid A, the major vertebrate acute-phase reactant. *European Journal of Biochemistry* **265**: 501-523.

UPRAGARIN, N. (2005) In vitro studies on the pathogenesis of AA amyloid arthropathy in chicken. *Ph. D. thesis,* Utrecht University.

UPRAGARIN, N., VAN ASTEN, A.J.A.M., TOOTEN, P.C.J., LANDMAN, W.J.M. and GRUYS, E. (2005) Serum amyloid A production by chicken fibroblast-like synoviocytes. *Veterinary Immunology and Immunopathology* **106**: 39-51.

WICHER, K.B. and FRIES, E. (2010) Evolutionary aspects of hemoglobin scavengers. *Antioxidants & Redox Signaling* **12**: 249-259.

WICHER, K.B. and FRIES, E. (2006) Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 4168-4173.

WIGLEY, P. and KAISER, P. (2003) Avian cytokines in health and disease. *Revista Brasileira de Ciência Avícola* **5**: 1-14.

WILLIAMS, J. (1968) A comparison of glycopeptides from the ovotransferrin and serum transferrin of the hen. *The Biochemical Journal* **108**: 57-67.

XIE, H., RATH, N.C., HUFF, G.R., HUFF, W.E. and BALOG, J.M. (2000) Effects of Salmonella typhimurium lipopolysaccharide on broiler chickens. *Poultry Science* **79**: 33-40.

XIE, H., HUFF, G.R., HUFF, W.E., BALOG, J.M., HOLT, P. and RATH, N.C. (2002a) Identification of ovotransferrin as an acute phase protein in chickens. *Poultry Science* **81**: 112-20.

XIE, H., NEWBERRY, L., CLARK, F.D., HUFF, W.E., HUFF, G.R., BALOG, J.M. and RATH, N.C. (2002b) Changes in Serum Ovotransferrin Levels in Chickens with Experimentally Induced Inflammation and Diseases Changes in Serum Ovotransferrin Levels in Chickens with Experimentally Induced Inflammation and Diseases. *Avian Diseases* **46**: 122-131.

XIE, H., HUFF, G.R., HUFF, W.E., BALOG, J.M. and RATH, N.C. (2002c) Effects of ovotransferrin on chicken macrophages and heterophil-granulocytes. *Developmental & Comparative Immunology* **26**: 805-815.

Appendix 2.1 (section 2.2.1.2)

Bradford reagent (5X concentrate)

- 100 mg Coomassie Brilliant Blue G-250
- 47 ml Methanol (100%)
- 100 ml Phosphoric Acid (85%)
- QS to 200 ml with H2O
- Dissolve Coomassie in the methanol first then add other ingredients.

Appendix 2.2 (section 2.2.1.3)

Ceruloplasmin assay

- Sodium acetate buffer: 20.8g sodium acetate (trihydrate) dissolved in 250ml dH2O, adjust pH to 6.3 with acetic acid
- Add 0.0615g of P-phenylenediamine (PDP)
- Cover with tin foil, light sensitive.

Appendix 2.3 (section 2.2.1.5)

Ovt competitive ELISA coating buffer

- 8.4g Sodium hydrogen carbonate dissolve into 500ml dH2O
- Dissolve conalbumin at concentration of 1µg/µl for coating wells

Appendix 2.4 (section 2.2.2)

Tris Buffered Saline (x10)

- Tris 22g
- NaCl 72g
- Dissolve in 1L dH2O and adjust to pH 7.6 with concentrated HCl

Transfer buffer (x1)

- Tris 6.06g
- Glycine 28.8g
- Dissolve in 1600mldH2O add 400ml methanol
- Check pH, do not adjust should be pH8.

Appendix 2.5 (section 2.3.1.2)

Spot	Name	Protein Identification*		Score	Theoretical		Matched	Sequence	Coverage %
		UniProt	Mascot Protein ID		Mass Da	PI			
1	PIT 54.- Gallus gallus	Q98TD1		562	52670	4.6	25(17)	12(10)	38
	Vitronectin.- Gallus gallus	O12945		121	52345	5.17	3(2)	2(1)	6
	Uncharacterized protein-(Predicted pantetheinase precursor [Gallus gallus]*)	Q5ZHM4		107	56105	6.33	3(3)	2(2)	5
	Serum albumin - chicken	P19121		73	71868	5.51	9(1)	8(1)	17
2	Alpha 1-acid glycoprotein - chicken	JC7879		212	22535	5.11	9(5)	6(5)	32
	PIT 54.- Gallus gallus (Chicken).	Q98TD1		144	52670	4.6	6(3)	6(3)	27
3	Apolipoprotein AIV.- Gallus gallus (Chicken).	O93601		47	40828	4.8	3(0)	3(0)	10
	PIT 54.- Gallus gallus (Chicken).	Q98TD1		42	52670	4.6	2(0)	2(0)	8
4	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11099	50	10993	6.78	1(1)	1(1)	16
5	transthyretin precursor - chicken	P27731	S17827	735	16356	5.11	24(15)	6(6)	62
	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11099	98	10993	4.72	1(1)	1(1)	16
	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	89	16496	8.84	2(2)	2(2)	17
6	transthyretin precursor - chicken	P27731	S17827	366	16356	5.11	9(4)	6(4)	62
	PIT 54.- Gallus gallus	Q98TD1		54	52670	4.6	1(1)	1(1)	2
7	transthyretin precursor - chicken	P27731	S17827	440	16356	5.11	14(8)	6(6)	62
	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	61	16496	8.84	2(1)	2(1)	14
8	apolipoprotein A-I precursor - chicken	P08250	LPCHA1	1045	30661	5.58	68(32)	17(8)	52
	IG LIGHT CHAIN V-J REGION (FRAGMENT).- Gallus gallus	*	AAA48870	87	10502	5.04	2(2)	2(2)	29
	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11099	68	10993	4.72	2(1)	2(1)	32
9	apolipoprotein A-I precursor - chicken	LPCHA1	P08250	389	30661	5.58	32(9)	15(6)	55
	IG LIGHT CHAIN V-J REGION (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48870	195	10502	5.04	7(3)	2(2)	39
	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11099	177	10993	4.72	6(3)	3(3)	45
10	retinol-binding protein precursor - chicken	P41263	I50675	232	22843	5.93	11(5)	9(4)	52
	apolipoprotein A-I precursor - chicken	P08250	LPCHA1	119	30661	5.58	13(3)	9(3)	33
	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11099	72	10993	4.72	1(1)	1(1)	16
11	IGL PROTEIN (FRAGMENT).- Gallus gallus	*	AAA48901	214	10422	8.17	8(5)	4(4)	66
	Immunoglobulin heavy chain variable region (Fragment).- Gallus gallus	*	BAA11105	159	10717	5.13	3(2)	3(2)	43
	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11099	157	10993	4.72	4(2)	3(2)	40
	apolipoprotein A-I precursor - chicken	P08250	LPCHA1	144	30661	5.58	13(4)	10(4)	36
	Ig light chain precursor V-J region - chicken (fragment)	*	A21177	135	22769	5.36	13(3)	5(2)	32

12	IGL PROTEIN (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48901	184	10422	8.71	5(3)	3(3)	44
	CHICKEN IG REARRANGED LAMBDA-CHAIN GENE 18D-2 V1J-REGION, PARTIAL CDS (FRAGMENT).- Gallus gallus	*	CAB25124	151	30661	5.58	4(3)	3(3)	43
	IG LIGHT CHAIN V-J REGION (FRAGMENT).- Gallus gallus	*	AAA48883	149	10238	8.00	4(2)	3(2)	43
13	Immunoglobulin lambda chain (Fragment).- Gallus gallus	*	BAB47289	138	11226	5.99	3(3)	2(2)	28
	Immunoglobulin heavy chain variable region (Fragment).- Gallus gallus	*	BAA11105	135	10717	4.72	4(3)	3(2)	45
	Phosphoglycerate mutase 1 (EC 5.4.2.1) (EC 5.4.2.4) (EC 3.1.3.13) (BPG-dependent PGAM 1).- Gallus gallus (Chicken).	Q5ZLN1	PGAM1	112	28920	7.21	5(2)	5(2)	30
	Ig light chain precursor V-J region - chicken (fragment)	*	A21177	111	22769	5.36	4(2)	4(2)	23
	Immunoglobulin lambda light chain (Fragment).- Gallus gallus (Chicken).	*	BAB71891	90	11175	9.00	2(2)	2(2)	28
	apolipoprotein A-I precursor - chicken	P08250	LPCHA1	70	30661	5.58	4(2)	4(2)	16
14	Immunoglobulin lambda light chain (Fragment).- Gallus gallus (Chicken).	*	BAB71911	163	11410	8.78	4(3)	3(3)	41
	AB233021 NID: - Gallus gallus (immunoglobulin light chain variable region)	*	BAE80151	155	12497	7.98	5(2)	3(2)	37
	CHICKEN IG REARRANGED LAMBDA-CHAIN GENE 18D-3 V1J-REGION, PARTIAL CDS (FRAGMENT).- Gallus gallus (Chicken)	*	CAB25125	138	10827	8.98	3(3)	3(3)	43
	IG LIGHT CHAIN V-J REGION (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48883	136	10238	8.00	3(3)	3(3)	50
	Immunoglobulin heavy chain variable region (Fragment).- Gallus gallus	*	BAA11105	109	10717	4.72	3(3)	3(3)	43
	apolipoprotein A-I precursor - chicken	P08250	LPCHA1	105	11410	8.78	9(2)	7(2)	21
15	apolipoprotein A-I precursor - chicken	P08250	LPCHA1	377	11410	8.78	9(4)	9(4)	35
	Ig light chain precursor V-J region - chicken (fragment)	*	A21177	219	22769	5.36	5(4)	4(3)	23
	CHICKEN IG REARRANGED LAMBDA-CHAIN GENE 3W-1 V1J-REGION, PARTIAL CDS (FRAGMENT).- Gallus gallus (Chicken).	*	CAB25129	167	10362	4.39	4(2)	3(2)	55
	Immunoglobulin heavy chain variable region (Fragment).- Gallus gallus	*	BAA11105	139	10717	4.72	3(2)	2(2)	30
16	Immunoglobulin lambda light chain (Fragment).- Gallus gallus (Chicken).	*	BAB71911	183	11410	8.78	4(4)	3(3)	41
	Ig light chain precursor V-J region - chicken (fragment)	*	A21177	165	22769	5.36	7(3)	4(3)	37
	AB233021 NID: - Gallus gallus (immunoglobulin light chain variable region)	*	BAE80151	122	12497	7.98	3(2)	2(2)	27
17	Ig light chain precursor V-J region - chicken (fragment)	*	A21177	254	22769	5.36	8(6)	5(4)	37
	Immunoglobulin lambda light chain (Fragment).- Gallus gallus (Chicken).	*	BAB71911	124	11410	8.78	4(2)	4(2)	48
	Immunoglobulin lambda chain variable region (Fragment).- Gallus gallus	*	BAA11101	111	10883	4.72	4(3)	3(2)	45
18	Apolipoprotein AIV.- Gallus gallus (Chicken).	O93601		1232	40828	4.8	37(20)	21(14)	60
19	transthyretin precursor – chicken	P27731	S17827	402	16356	5.11	10(9)	6(6)	62
20	Hemopexin (Fragment).- Gallus gallus (Chicken).	Q90WR3		438	29765	5.92	15(10)	9(6)	52

21	serum albumin – chicken	P19121	ABCHS	1206	71868	5.51	66(32)	32(20)	62
	Vitronectin.- Gallus gallus	O12945		89	52345	5.17	3(2)	3(2)	11
	Immunoglobulin heavy chain (Fragment).- Gallus gallus (Chicken).	*	BAA10003	64	12975	4.97	1(1)	1(1)	9
24	serum albumin – chicken	P19121	ABCHS	1595	71868	5.51	80(42)	33(20)	64
	GGIGMUCH NID: - Gallus gallus (Ig mu chain C region)	*	CAA25762	177	48827	6.07	7(4)	5(3)	15
	ovoinhibitor precursor [validated] – chicken	P10184	A26730	100	54394	6.16	8(2)	7(2)	21
	IG HEAVY-CHAIN V REGION (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48835	60	11933	7.88	2(1)	2(1)	29
25	serum albumin – chicken	P19121	ABCHS	1404	71868	5.51	73(33)	31(19)	58
	serum albumin – chicken	P19121	ABCHS	486	71868	5.51	17(4)	16(3)	34
30	Mannose-binding lectin precursor protein.- Gallus gallus (Chicken).	Q98TA4		143	27758	5.85	5(1)	4(1)	21
31	Ovotransferrin BB type.- Gallus gallus (Chicken).	Q4ADJ7		1018	79606	6.85	48(24)	31(17)	52
	ovotransferrin– chicken	P02789	TFCHE	906	79551	6.85	41(21)	27(16)	51
33	Ovotransferrin BC type.- Gallus gallus (Chicken).	Q4ADG4		1704	79588	7.08	83(39)	35(20)	57
	ovotransferrin – chicken	P02789	TFCHE	1699	79551	6.85	81(39)	34(20)	56
34	Ovotransferrin BC type.- Gallus gallus (Chicken).	Q4ADG4		989	79588	7.08	57(23)	32(18)	55
35	Ovotransferrin BC type.- Gallus gallus (Chicken).	Q4ADG4		298	79588	7.08	11(4)	11(4)	23
	ovotransferrin– chicken	P02789	TFCHE	297	79551	6.85	10(4)	10(4)	23
	Ig gamma chain (clone 36) - chicken (fragment)	*	S00390	77	54436	6.84	4(0)	4(0)	13
36	Ig gamma chain (clone 36) - chicken (fragment)	*	S00390	180	54436	6.84	6(4)	5(4)	15
	IG HEAVY-CHAIN V REGION (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48844	148	11878	7.86	4(2)	4(2)	50
37	Ig gamma chain (clone 36) - chicken (fragment)	*	S00390	312	54436	6.84	20(6)	11(6)	28
	Immunoglobulin heavy chain variable region (Fragment).- Gallus gallus (Chicken).	*	BAA11100	147	13188	8.44	4")	3(2)	33
	IG HEAVY-CHAIN V REGION (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48844	130	11878	7.86	5(2)	4(2)	42
40	Ig gamma chain (clone 36) - chicken (fragment)	*	S00390	198	54436	6.84	12(5)	7(5)	16
	IG HEAVY-CHAIN V REGION (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48837	168	11894	8.45	4(3)	2(3)	42
41	fibrinogen beta chain - chicken (fragment)	Q02020	A38463	858	53272	7.18	70(23)	27(16)	68
	GGFIBA5 NID: - Gallus gallus (pre-fibrinogen alpha subunit [Gallus gallus])	P14448	AAB60685	177	56766	6.82	9(5)	7(4)	18
	Vitamin-D binding protein.- Gallus gallus (Chicken).	Q9W6F5		110	55362	6.47	7(2)	7(2)	19
42	fibrinogen beta chain - chicken (fragment)	Q02020	A38463	1184	53272	7.18	47(18)	27(15)	68
	GGFIBA5 NID: - Gallus gallus(pre-fibrinogen alpha subunit [Gallus gallus])	P14448	AAB60685	820	56766	6.82	22(14)	16(10)	45
44	fibrinogen beta chain - chicken (fragment)	Q02020	A38463	828	53272	7.18	26(8)	20(7)	45
45	GGFIBA5 NID: - Gallus gallus(pre-fibrinogen alpha subunit [Gallus gallus])	P14448	AAB60685	829	56766	6.82	31(19)	12(12)	57

	Ig gamma chain (clone 36) - chicken (fragment)		S00390	63	54436	6.84	3(2)	2(1)	6
46	fibrinogen beta chain - chicken (fragment)	Q02020	A38463	840	53272	7.18	59(23)	26(16)	60
	GGFIBA5 NID: - Gallus gallus(pre-fibrinogen alpha subunit [Gallus gallus])	P14448	AAB60685	216	56766	6.82	11(7)	8(6)	21
	Vitamin-D binding protein.- Gallus gallus (Chicken).	Q9W6F5		120	55362	6.47	8(2)	8(2)	20
47	Vitamin-D binding protein.- Gallus gallus (Chicken).	Q9W6F5		402	55362	6.47	23(8)	17(6)	47
	ovoinhibitor precursor [validated] - chicken	P10184	A26730	127	54394	6.16	14(3)	9(3)	20
	fibrinogen beta chain - chicken (fragment)	Q02020	A38463	125	53272	7.18	8(4)	8(4)	20
48	GGFIBA5 NID: - Gallus gallus(pre-fibrinogen alpha subunit [Gallus gallus])	P14448	AAB60685	139	56766	6.82	4(2)	4(2)	11
49	Trypsin/Keratin (no Gallus gallus protein)								
50	Trypsin/Keratin (no Gallus gallus protein)								
51	keratin								
52	chicken hemoglobin d (EC 2.3.1.61), chain C - chicken	P02001	1HBRC	835	15586	6.58	35(22)	9(5)	82
	Hemoglobin subunit alpha-A (Hemoglobin alpha-A chain) Gallus gallus (Chicken).	P01994	HBA_CHICK	580	15402	8.56	20(11)	8(5)	65
	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	136	16496	8.85	10(4)	8(3)	66
	hemoglobin beta-H chain - chicken	Q90864	I50248	109	16469	9.20	6(2)	4(1)	29
	cystatin precursor - chicken	P01038	UDCH	56	15562	7.60	2(1)	2(1)	20
53	Hemoglobin subunit alpha-A (Hemoglobin alpha-A chain) Gallus gallus (Chicken).	P01994	HBA_CHICK	469	15402	8.56	14(8)	6(5)	51
	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	242	16496	8.85	19(7)	9(3)	73
	chicken hemoglobin d (EC 2.3.1.61), chain C - chicken	P02001	1HBRC	104	15586	6.58	4(3)	3(2)	37
54	Hemoglobin subunit alpha-A (Hemoglobin alpha-A chain) Gallus gallus (Chicken).	P01994	HBA_CHICK	613	15402	8.56	30(16)	10(6)	89
	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	197	16496	8.85	9(5)	7(5)	61
	serum albumin – chicken	P19121	ABCHS	157	71868	5.51	14(4)	12(4)	22
55	Hemoglobin subunit alpha-A (Hemoglobin alpha-A chain) Gallus gallus (Chicken).	P01994	HBA_CHICK	355	15402	8.56	12(7)	7(5)	60
	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	302	16496	8.85	27(10)	11(6)	78
	hemoglobin beta-H chain - chicken	Q90864	I50248	173	16469	9.20	19(4)	8(2)	46
56	Hemoglobin subunit beta (Hemoglobin beta chain) (Beta-globin).- Gallus gallus	P02112	HBB_CHICK	107	16496	8.85	4(1)	4(1)	39
	Hemoglobin subunit alpha-A (Hemoglobin alpha-A chain) Gallus gallus (Chicken).	P01994	HBA_CHICK	90	15402	8.56	2(1)	2(1)	19
58	serum albumin – chicken	P19121	ABCHS	263	71868	5.51	8(1)	8(1)	15
	IGL PROTEIN (FRAGMENT).- Gallus gallus (Chicken).	*	AAA48901	235	10422	8.71	4(4)	3(3)	44
	Ig light chain precursor V-J region - chicken (fragment)	*	A21177	212	22769	5.36	4(3)	3(3)	23
	Immunoglobulin lambda light chain (Fragment).- Gallus gallus (Chicken).	*	BAB71891	210	11175	9.00	3(3)	3(3)	40
	Immunoglobulin heavy chain variable region (Fragment).- Gallus gallus	*	BAA11105	201	10717	5.13	3(3)	3(3)	43

*Uniprot Accessions are given. Where Mascot identifier is different this is listed on the right. Note that Immunoglobulins are not listed in UniProt. The Mascot link reference can be

opened in UniParc, the UniProt archive.
****Mascot online database of results has deleted/moved this protein and original result of coverage not available.**

Bands were excised from the gel trypsin digested and identified using nanoflow HPLC-EPI-MS/MS with identities assigned using Mascot Science Daemon server to interrogate protein sequences in the NCBI Genbank database for *Gallus gallus* and boney vertebrates. Both NCBI and where Uniprot identifications are listed (immunoglobulins are not listed in Uniprot). The protein score is the sum of the highest ions score for each distinct sequence. The first number under matches is the count of MS/MS spectra that have been matched to peptides from this protein. The matches shown in the parenthesis indicating unique matches (scores above the significance threshold). The sequences column is the count of matches to distinct peptide sequences, the number in parenthesis is the count of sequences with significant matches (Matrixscience.com). The coverage percentage is proportion of the protein's entire sequence identified.

Appendix 3.1 (section 3.2)

IEP buffer

- Tris 9.8g
- Calcium lactate 0.106g
- Sodium azide 0.2g
- Tricine 4.3g
- Dissolve in 1L dH2O

RID gel

- Dissolve 0.17g agarose in 17ml TBS
- While in water bath (56˚C) add antibody, mix and pour immediately

Appendix 4.1 (section 4.2.1.1)

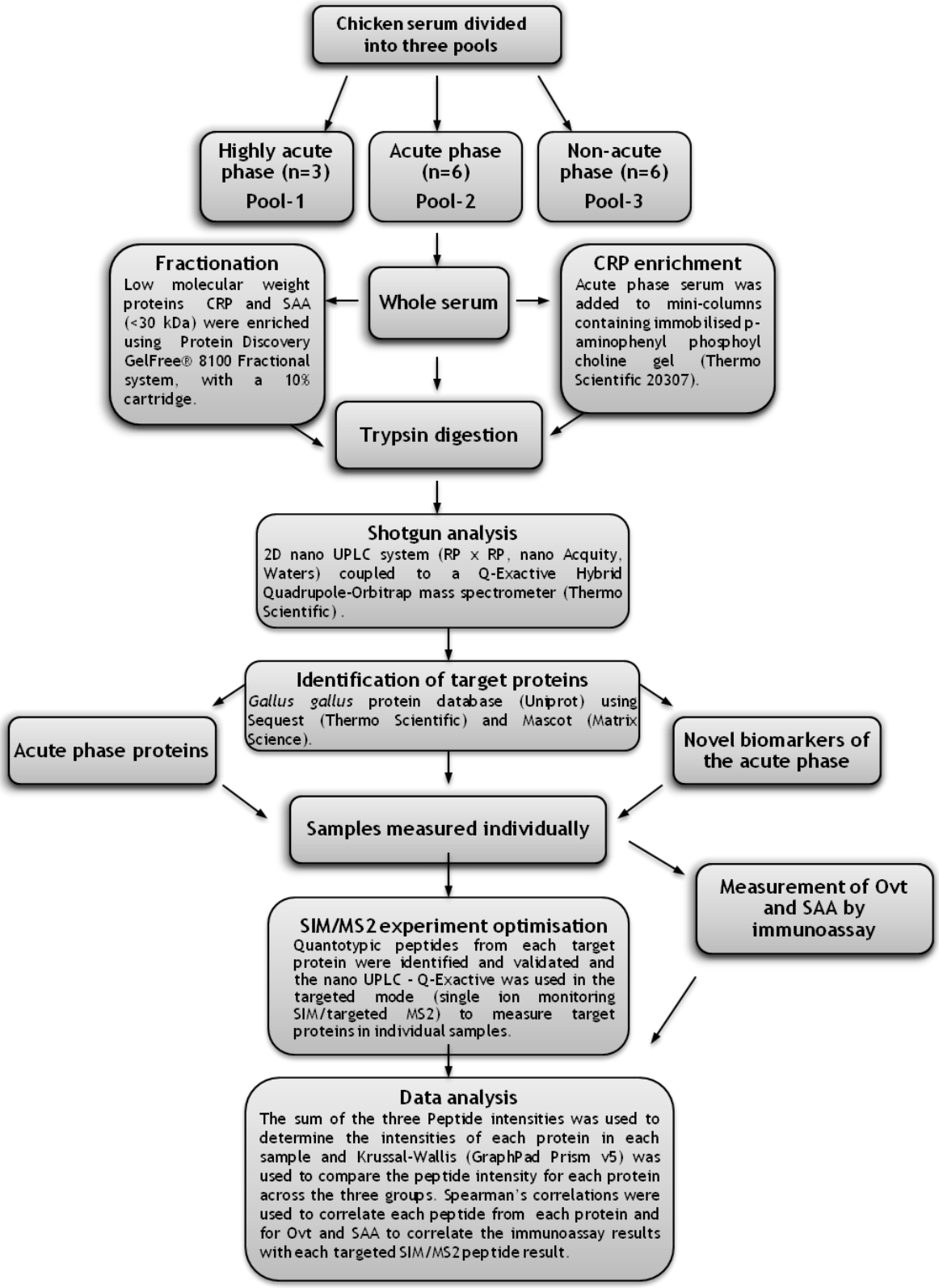
CRP Binding buffer

- Tris 12.114g
- NaCl 5.844
- CaCl2 0.22g
- pH 8-8.5

CRP Elution buffer

- Tris 12.114
- NaCl 5.884
- EDTA 0.586g
- pH 8-8.5

Appendix 4.2 (section 4.2.2.2.2)



Work flow chart summarising the work process of Chapter 4.

Appendix 4.3 (section 4.3.2.2.1)

Pools	Protein	Accession	Fold change
High acute phase: Acute phase	Uncharacterized protein	E1BZE1	-44.05
	Uncharacterized protein	E1BS56	-26.57
	Uncharacterized protein	R4GJ67	-20.06
	Vinculin (Metavinculin)	P12003	-19.70
	Uncharacterized protein	F1NKD4	-18.78
	Matrilin-3	O42401	-18.41
	Uncharacterized protein	F1P4C5	-9.22
	Uncharacterized protein	F1P4N9	-8.59
	Uncharacterized protein	E1C6J4	-8.12
	Uncharacterized protein	F1NEQ4	-8.06
	Neuronal-glial cell adhesion molecule (Ng-CAM)	Q03696	-7.96
	Uncharacterized protein	E1C7H6	-6.79
	Uncharacterized protein (Fragment)	F1NUF8	-6.35
	Lumican (Keratan sulfate proteoglycan lumican) (KSPG lumican)	P51890	-6.21
	Uncharacterized protein	E1C544	-5.02
	Uncharacterized protein (Fragment)	F1NRM4	-4.85
	Uncharacterized protein	F1NHT5	-4.63
	Protein NEL (93 kDa protein)	Q90827	4.04
	Fructose-bisphosphate aldolase (EC 4.1.2.13)	R4GM10	4.07
	Uncharacterized protein	R4GMH4	4.16
	Non-specific serine/threonine protein kinase (EC 2.7.11.1)	E1BWY7	4.64
	Uncharacterized protein	F1N8D4	5.10
	Uncharacterized protein (Fragment)	F1NSD3	5.20
	Uncharacterized protein (Fragment)	F1NLZ2	5.36
	Uncharacterized protein (Fragment)	F1NXB6	5.39
	14-3-3 protein theta	Q5ZMD1	5.71
	Uncharacterized protein	F1P2W2	6.24
	Uncharacterized protein	R4GG01	7.31
	Docking protein 3 (Downstream of tyrosine kinase 3)	A3R064	8.02
	Uncharacterized protein (Fragment)	F1NJD5	8.59
	Uncharacterized protein	F1NTF4	10.33
	Tenascin (TN) (Cytotactin)	P10039	10.61
	Clusterin (51.5 kDa protein)	P14018	10.62
	Uncharacterized protein	E1BV78	11.38
	Beta-2-microglobulin	P21611	11.76
	L-lactate dehydrogenase B chain (LDH-B) (EC 1.1.1.27)	P00337	17.18
	Sixth complement component (Uncharacterized protein)	B8ZX71	18.89
	Fibrinogen alpha chain [Cleaved into: Fibrinopeptide A;	P14448	21.34
Acute phase - Non-acute phase	Putative ISG12(2) protein (Uncharacterized protein)	Q6IEC5	-42.70
	Malate dehydrogenase, cytoplasmic (EC 1.1.1.37) (Cytosolic malate	Q5ZME2	-12.79
	Lamin-A	P13648	-11.70
	Uncharacterized protein (Fragment)	F1NJD5	-9.86
	Docking protein 3 (Downstream of tyrosine kinase 3)	A3R064	-9.78
	Uncharacterized protein	E1C1F2	-9.50
	Uncharacterized protein	E1C8B4	-7.60
	Uncharacterized protein	E1BWG1	-6.58
	Cadherin-5 (Vascular endothelial cadherin)	F1P1Y9	-6.49
	Uncharacterized protein (Fragment)	F1NLZ2	-5.87
	Serum albumin (BSA) (allergen Bos d 6)	P02769	-4.78
	Uncharacterized protein	R4GMH4	-4.34
	Uncharacterized protein	F1N869	4.45
	Uncharacterized protein (Fragment)	F1NAB7	5.20
	Keratin, type I cytoskeletal 9	P35527	5.76
	L-lactate dehydrogenase A chain (LDH-A) (EC 1.1.1.27)	P00340	6.15
	Neuronal-glial cell adhesion molecule (Ng-CAM)	Q03696	6.98
	Uncharacterized protein (Fragment)	F1NCH8	7.09
	Uncharacterized protein	E1C6J4	7.93
	Uncharacterized protein	R4GJ67	9.20
	Plasminogen (EC 3.4.21.7)	R4GMH5	10.73

	Uncharacterized protein	F1NNW0	13.08
	Beta-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase)	P07322	14.12
	Cathepsin D (EC 3.4.23.5)	Q05744	22.64
	Coronin	Q5ZI60	59.85
High acute phase: Non-acute phase	Uncharacterized protein	E1BZE1	-52.65
	Matrilin-3	O42401	-20.99
	Putative ISG12(2) protein (Uncharacterized protein)	Q6IEC5	-18.39
	Uncharacterized protein	E1C1F2	-15.28
	Uncharacterized protein	F1NKD4	-14.98
	Uncharacterized protein	F1P4N9	-14.23
	Cadherin-5 (Vascular endothelial cadherin) (VE-cadherin)	F1P1Y9	-11.54
	Uncharacterized protein	F1NEQ4	-11.14
	Uncharacterized protein	R4GGS2	-10.97
	Uncharacterized protein	E1BWG1	-10.94
	Uncharacterized protein	E1BS56	-10.76
	Ovocleidin-116 (OC-116)	F1NSM7	-10.76
	Uncharacterized protein	F1NYP5	-9.02
	Uncharacterized protein	E1BWN2	-8.30
	Histone-lysine N-methyltransferase SETD1B (EC 2.1.1.43)	Q5F3P8	-7.69
	Vinculin (Metavinculin)	P12003	-6.88
	Serum albumin (BSA) (allergen Bos d 6)	P02769	-6.53
	Uncharacterized protein	E1C8B4	-5.66
	Uncharacterized protein	E1BX43	-5.42
	Uncharacterized protein (Fragment)	F1NSF6	-5.42
	Uncharacterized protein	F1P4C5	-5.36
	Malate dehydrogenase, cytoplasmic (EC 1.1.1.37)	Q5ZME2	-5.31
	Uncharacterized protein	F1NHT5	-5.13
	Uncharacterized protein	E1BYT9	-4.88
	Lamin-A	P13648	-4.87
	Uncharacterized protein (Fragment)	F1NUF8	-4.43
	Uncharacterized protein	E1BTX1	4.28
	Uncharacterized protein	E1C7M0	4.49
	Uncharacterized protein	F1NTF4	4.64
	Ribonuclease homolog (EC 3.1.27.-) (RSFR)	P30374	4.74
	Uncharacterized protein	F1NDJ8	4.79
	Uncharacterized protein	F1P2W2	5.14
	Beta-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase)	P07322	5.33
	Uncharacterized protein	F1NNW0	5.62
	L-lactate dehydrogenase A chain (LDH-A) (EC 1.1.1.27)	P00340	5.99
	Uncharacterized protein (Fragment)	F1NSD3	6.11
	Uncharacterized protein	F1P587	6.42
	Uncharacterized protein	E1C4F1	6.51
	Uncharacterized protein	E1BV78	7.89
	Plasminogen (EC 3.4.21.7)	R4GMH5	8.17
	Uncharacterized protein	R4GG01	9.42
	Tenascin (TN) (Cytotactin)	P10039	10.39
	Clusterin (51.5 kDa protein)	P14018	13.17
	Cathepsin D (EC 3.4.23.5)	Q05744	17.65
	Uncharacterized protein (Fragment)	F1NAB7	18.25
	Fibrinogen alpha chain	P14448	20.48
	L-lactate dehydrogenase B chain (LDH-B) (EC 1.1.1.27)	P00337	21.31
	Sixth complement component (Uncharacterized protein)	B8ZX71	22.91
	Beta-2-microglobulin	P21611	25.68
	Coronin	Q5ZI60	25.89

Proteins identified in all three acute phase pools (pools 1-3), ranked by fold difference between pools and excluding proteins with less than 4 fold difference. Highlighted proteins were selected for further measurement in section 4.3.2.2.2.

Appendix 4.4 (section 4.3.2.2.1)

Protein ID	Accession	Fold change
Uncharacterized protein	E1C932	-61.56
Uncharacterized protein	R4GL55	-7.16
Uncharacterized protein	R4GHQ6	-6.62
Retinoid isomerohydrolase (EC 3.1.1.64) (All-trans-retinyl-palmitate hydrolase) (Retinal pigment epithelium-specific 65 kDa protein) (Retinol isomerase)	Q9YGX2	-5.89
Uncharacterized protein	F1NAP7	-5.89
Signal recognition particle subunit SRP68 (SRP68)	F1P0P4	-4.71
Uncharacterized protein	F1NGF6	-3.47
Hsp90 co-chaperone Cdc37 (Hsp90 chaperone protein kinase-targeting subunit) (p50Cdc37)	O57476	-3.14
Uncharacterized protein	F1NJN2	5.37
Serum amyloid A protein*	F1NW65	5.62
Ras-responsive element-binding protein 1 (RREB-1)	O57415	6.21

Proteins present only in acute phase groups (pools 1 and 2) that show more than a 4 fold difference between these pools. Highlighted proteins were selected for further measurement in section 4.3.2.2.2, with the exception of serum amyloid A which was measured in section 4.3.2.1.2.

Appendix 5.1 (section 5.3.3.2)

Apo-AIV indirect ELISA coating buffer

- 50mM sodium hydrogen cardonate
- 4.2g into 1L dH2O, pH 9

TBS-tween 0.05% (stock): 1L TBS + 0.5ml tween

TBS-tween 0.05%/5% Marvel (blocking): 100ml TBS-tween0.05%+5g Marvel

TBS-tween 0.05%/1% Marvel (washing): 1L TBS-tween (0.05%) + 10g Marvel

TBS-1% Marvel (diluting antibodies): 100ml TBS + 1g Marvel

Appendix 6.1 (Section 6.2.4)

DiGE Lysis buffer

- 6M urea
- 2M thiourea
- 4% CHAPS
- 25mM tris base

Equilibration buffer I

- 1.5M Tris HCl
- 216.21 g urea
- 180ml glycerol
- 12g SDS
- 100mg DTT
- pH8.8

Equilibration buffer 2

- 1.5M Tris HCl
- 216.21 g urea
- 180ml glycerol
- 12g SDS 280
- pH8.8

References

Abdallah, C., Dumas-Gaudot, E., Renaut, J., & Sergeant, K. (2012). Gel-based and gel-free quantitative proteomics approaches at a glance. *International Journal of Plant Genomics*. **2012**, Article ID 494572.

Adler, K. L., Peng, P. H., Peng, R. K., Klasing, K. C., & Klasinga, K. C. (2001). The Kinetics of Hemopexin and α1-Acid Glycoprotein Levels Induced by Injection of Inflammatory Agents in Chickens. *Avian Diseases*. **45**, 289–296.

Aguilera, O., Quiros, L. M., & Fierro, J. F. (2003). Transferrins selectively cause ion efflux through bacterial and artificial membranes. *FEBS Letters*. **548**, 5–10.

Alasonyalilar, A., Sevimli, A., Misirlioglu, D & Uguz, C. (2006). Chronic amyloid arthropathy and increased serum amyloid levels in brown layers. *The Bulletin of the Veterinary Institute in Pulawy*. **50**, 557–560.

Alban, A., David, S. O., Bjorkesten, L., Andersson, C., Sloge, E., Lewis, S., & Currie, I. (2003). A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics*. **3**, 36–44.

Alicea, H. A. & Mooseker, M. S. (1988). Characterization of villin from the intestinal brush border of the rat, and comparative analysis with avian villin. *Cell Motililty and the Cytoskeleton*. **9**, 60–72.

Almeida, A. M., Bassols, A., Bendixen, E., Bhide, M., Ceciliani, F., Cristobal, S., … Turk, R., Eckersall, P. D., Hollung, K., Lisacek, F., Mazzucchelli, G., McLaughlin, M., Miller, I., Nally, J. E., Plowman, J., Renaut, J., Rodrigues, P., Roncada, P., Staric, J. & Turk, R (2015). Animal board invited review: advances in proteomics for animal and food sciences. *Animal : An International Journal of Animal Bioscience*. **9**, 1–17.

Amrani, D. L., Mauzy-Melitz, D., & Mosesson, M. W. (1986). Effect of hepatocyte-stimulating factor and glucocorticoids on plasma fibronectin levels. *The Biochemical Journal*. **238**, 365–71.

Arentz, G., Weiland, F., Oehler, M. K. & Hoffmann, P. (2015). State of the art of 2D DIGE. *Proteomics - Clinical Applications*. **9**, 277–288.

Arike, L., & Peil, L. (2014). Spectral Counting Label-Free Proteomics. *Methods in Molecular Biology*. **1156**, 213–222.

Asasi, K., Mohammadi, A., Boroomand, Z., Hosseinian, S. A., & Nazifi, S. (2013). Changes of several acute phase factors in broiler chickens in response to infectious bronchitis virus infection. *Poultry Science*. **92**, 1989–1996.

Ascenzi, P., Bocedi, A., Visca, P., Altruda, F., Tolosano, E., Beringhelli, T., & Fasano, M. (2005). Hemoglobin and heme scavenging. *IUBMB Life*. **57**, 749–59.

Athman, R., Louvard, D., & Robine, S. (2002). The epithelial cell cytoskeleton and intracellular trafficking. III. How is villin involved in the actin cytoskeleton dynamics in intestinal cells. *American Journal of Physiology, Gastrointestinal and Liver Physiology*. **283**, 496–502.

Awadé, A. C., Moreau, S., Mollé, D., Brulé, G., & Maubois, J. L. (1994). Two-step chromatographic procedure for the purification of hen egg white ovomucin, lysozyme, ovotransferrin and ovalbumin and characterization of purified proteins. *Journal of Chromatography*. **677**, 279–88.

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*. **72**, 4–15.

Barle, H., Nyberg, B., Essén, P., Andersson, K., McNurlan, M. A, Wernerman, J., & Garlick, P. J. (1997). The synthesis rates of total liver protein and plasma albumin determined simultaneously in vivo in humans. *Hepatology*. **25**, 154–8.

Barnes, D. M., Song, Z., Klasing, K. C., & Bottje, W. (2002). Protein metabolism during an acute phase response in chickens. *Amino Acids*. **22**, 15–26.

Bennett, D., Eckersall, P. D., Waterston, M., Marchetti, V., Rota, A., McCulloch, E., & Sbrana, S. (2013). The effect of robenacoxib on the concentration of C-reactive protein in synovial fluid from dogs with osteoarthritis. *BMC Veterinary Research*. **9**, 1-11.

Berbée, J. F. P., Havekes, L. M., & Rensen, P. C. N. (2005). Apolipoproteins modulate the inflammatory response to lipopolysaccharide. *Journal of Endotoxin Research*. **11**, 97–103.

Blake, D. P., & Tomley, F. M. (2014). Securing poultry production from the ever-present Eimeria challenge. *Trends in Parasitology*. **30**, 12–19.

Bon, E., Steegers, R., Steegers, E. A. P., Ursem, N., Charif, H., Burgers, P. C., Luider, T. M., & Dekker, L. J. M. (2010). Proteomic analyses of the developing chicken cardiovascular system. *Journal of Proteome Research*. **9**, 268–274.

Butler, E. J., Curtis, M. J.; Harry, E. G., & Deb, J. R. (1972). Effects of Escherichia coli endotoxins on plasma para-phenylenediamine oxidase (caeruloplasmin) activity in the domestic fowl. *Journal of Comparative Pathology*. **82**, 299–306.

Buyse, J., Swennen, Q., Niewold, T. A., Klasing, K. C., Janssens, G. P. J., Baumgartner, M., & Goddeeris, B. M. (2007). Dietary L-carnitine supplementation enhances the lipopolysaccharide-induced acute phase protein response in broiler chickens. *Veterinary Immunology and Immunopathology*. **118**, 154–9.

Campbell, F. M., Makala, L. H., Mangani, P., Fujisaki, K., & Nagasawa, H. (2005). The negative acute response of serum transthyretin following Streptococcus suis infection in the pig. *Veterinary Research*. **36**, 657–664.

Carpintero, R., Alonso, C., Piñeiro, M., Iturralde, M., Andrés, M., Le Potier, M. F., Madec, F., Álava, M. Á., Piñeiro, A., & Lampreave, F. (2007). Pig major acute-phase protein and apolipoprotein A-I responses correlate with the clinical course of experimentally induced African swine fever and Aujeszky's disease. *Veterinary Research*. **38**, 741–753.

Carpintero, R., Piñeiro, M., Andrés, M., Iturralde, M., Alava, M. A., Heegaard, P. M. H., Jobert, J. L., Madec, F., & Lampreave, F. (2005). The concentration of apolipoprotein A-I decreases during experimentally induced acute-phase processes in pigs. *Infection and Immunity*. **73**, 3184–7.

Ceciliani, F., Eckersall, D., Burchmore, R., & Lecchi, C. (2014). Proteomics in veterinary medicine: applications and trends in disease pathogenesis and diagnostics. *Veterinary Pathology*. **51**, 351–62.

Ceciliani, F., & Pocacqua, V. (2007). The acute phase protein 1-acid glycoprotein: a model for altered glycosylation during diseases. *Current Protein and Peptide Science*. **8**, 91–108.

Ceron, J. J., Eckersall, P. D., & Martýnez-Subiela, S. (2005). Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Veterinary Clinical Pathology*. **34**, 85–99.

Chamanza, R., Toussaint, M., van Ederen, A. M., van Veen, L., Hulskamp-Koch, C., & Fabri, T. H. F. (1999). Serum amyloid a and transferrin in chicken. A preliminary investigation of using acuteâ€¤phase variables to assess diseases in chickens. *Veterinary Quarterly*. **21**, 37–41.

Chang, L., Munro, S. L. A, Richardson, S. J., & Schreiber, G. (1999). Evolution of thyroid hormone binding by transthyretins in birds and mammals. *European Journal of Biochemistry*. **259**, 534–542.

Chen, W. T., Wu, Y. L., Chen, T., Cheng, C. S., Chan, H. L., Chou, H. C., Chen, Y. W., & Yin, H. S. (2014). Proteomics analysis of the DF-1 chicken fibroblasts infected with avian reovirus strain S1133. *PLoS ONE*. **9**, 1–10.

Cheriyath, V., Leaman, D. W., & Borden, E. C. (2011). Emerging roles of FAM14 family members (G1P3/ISG 6-16 and ISG12/IFI27) in innate immunity and cancer. *Journal of Interferon & Cytokine Research*. **31**, 173–181.

Cignarelli, M., DePergola, G., Picca, G., Sciaraffia, M., Pannacciulli, N., Tarallo, M., Laudadio, E., & Turrisi E, G. R. (1996). Relationship of obesity and body fat distribution with ceruloplasmin serum levels. *Journal of the International Association for the Study of Obesity*. **20**, 809–813.

Cohly, H. H. P., Scott, H., Ndebele, K., Jenkins, J. K., & Angel, M. F. (2002). Differential Gene Expression of Fibroblasts: Keloid versus Normal. *International Journal of Molecular Sciences*. **3**, 1162–1176.

Cooper, E. H., & Plesner, T. (1980). Beta-2-Microglobulin Review: Its Relevance in Clinical Oncology. *Medical and Pediatric Oncology*. **334**, 323–334.

Cray, C., Zaias, J., & Altman, N. H. (2009). Acute phase response in animals: a review. *Comparative Medicine*. **59**, 517–26.

Crespo, R., & Shivaprasad, H. L. (2011). Rupture of Gastrocnemius Tendon in Broiler Breeder Hens. *Avian Diseases*. **55**, 495–498.

Cretu, D., Liang, K., Saraon, P., Batruch, I., Diamandis, E. P., & Chandran, V. (2015). Quantitative tandem mass-spectrometry of skin tissue reveals putative psoriatic arthritis biomarkers. *Clinical Proteomics*. **12**, 1–8.

Dabrowska, A. M., Tarach, J. S., Wojtysiak-Duma, B., & Duma, D. (2015). Fetuin-A (AHSG) and its usefulness in clinical practice - review of the literature. *Biomedical Papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia*. **159**, 352-359.

Dalgaard, T. S., Skovgaard, K., Norup, L. R., Pleidrup, J., Permin, A., Schou, T. W., Vadekær, D. F., Jungersen, G., & Juul-Madsen, H. R. (2015). Immune gene expression in the spleen of chickens experimentally infected with Ascaridia galli. *Veterinary Immunology and Immunopathology*. **164**, 79–86.

Davalos, D. & Akassoglou, K.,(2012). Fibrinogen as a key regulator of inflammation in disease. *Seminars in immunopathology*, **34**, 43–62.

de Hostos, E. L. (1999). The coronin family of actin-associated proteins. *Trends in Cell Biology*. **9**, 345–350.

Dean, R. T. (1975). Direct evidence of importance of lysosomes in degradation of intracellular proteins. *Nature*. **257**, 414–416.

Degn, S. E., Jensen, L., Hansen, A. G., Duman, D., Tekin, M., Jensenius, J. C., & Thiel, S. (2012). MASP-1 is crucial for lectin pathway activation in human serum, while neither MASP-1 nor MASP-3 are required for alternative pathway function. *Immunobiology*. **217**, 1218–1219.

Delers, F., Musquera, S., Domingo, M., & Lombart, C., (1983). Characterization of a chicken hemoglobin-binding protein: A novel haptoglobin. *Comparative Biochemistry and Physiology -- Part A: Physiology*. **74**, 745–748.

Deng, X., Cong, Y., Yin, R., Yang, G., Ding, C., Yu, S., & Liu, X. (2014). Proteomic analysis of chicken peripheral blood mononuclear cells after infection by Newcastle disease virus. *Science*. **15,** 511–517.

Diack, A. B., Gladney, C. D., Mellencamp, M. a., Stear, M. J., & Eckersall, P. D. (2011). Characterisation of plasma acute phase protein concentrations in a high health boar herd. *Veterinary Immunology and Immunopathology*. **139**, 107–112.

Dinev, I. (2008). Clinical and morphological studies on spontaneous rupture of the gastrocnemius tendon in broiler breeders. *British Poultry Science*. **49**, 7–11.

Dinev, I. (2012). Leg Weakness Pathology in Broiler Chickens. *The Journal of Poultry Science*. **49**, 63–67.

Disilvestro, R. A, & Harris, E. D. (1985). Purification and partial characterization of ceruloplasmin from chicken serum. *Archives of Biochemistry and Biophysics*. **241**, 438–46.

Doherty, M. K., McLean, L., Hayter, J. R., Pratt, J. M., Robertson, D. H. L., El-Shafei, A., Gaskell, S. J., & Beynon, R. J. (2004). The proteome of chicken skeletal muscle: changes in soluble protein expression during growth in a layer strain. *Proteomics*. **4**, 2082–93.

Dudouet, B., Jacob, L., & Beuzeboc, P. (1990). Presence of villin, a tissue-specific cytoskeletal protein, in sera of patients and an initial clinical evaluation of its value for the diagnosis and follow-up of colorectal. *Cancer Research*. **50**, 438–443.

Duff, S. R. I., & Anderson, I. A. (1986). The gastrocnemius tendon of broiler fowls: histoligical findings in different strains. *Research in Veterinary Science*. **41**, 402–409.

Duff, S. R. I., & Randall, C. J. (1986). Tendon lesions in broiler fowls. *Research in Veterinary Science*. **40**, 333–338.

Engvall, E., & Perlmann, P., (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*. **8**, 871–874.

Eberini, I., Agnello, D., Miller, I., Fratelli, M., Ghezzi, P., Gemeiner, M., Chan, J., Gianazza, E.,

Ricerche, I., Mario, F., & Studi, U. (2000). Proteins of rat serum V : Adjuvant arthritis and its modulation by nonsteroidal anti-inflammatory drugs. Electrophoresis 2000. **21**, 2170–2179.

Eckersall, P. D., & Bell, R. (2010). Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. *Veterinary Journal*. **185**, 23–7.

Eckersall, P. D., Duthie, S., Safi, S., Moffatt, D., Horadagoda, N. U., Doyle, S., Parton, R., Bennett, D., & Fitzpatrick, J., (1999). An Automated Biochemical Assay for Haptoglobin : Prevention of Interference from Albumin. *Comparative Haematology International*. **9**, 117–124.

Eguchi, R., Ishihara, A., & Yamauchi, K. (2008). Interaction of diethylstilbestrol and ioxynil with transthyretin in chicken serum. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology*. **147**, 345–50.

Ekmay, R. D., de Beer, M., Rosebrough, R. W., Richards, M. P., McMurtry, J. P., & Coon, C. N. (2010). The role of feeding regimens in regulating metabolism of sexually mature broiler breeders. *Poultry Science*. **89**, 1171–1181.

Eriksen, N., Meek, R. L., & Benditt, E. P. (1993). The SAA lipoprotein family. In Mackiewicz , A., Kushner, I., & Baumann, H. ed. (1993) *Acute Phase Proteins: Molecular biology, Biochemistry and Clinical Applications*. London: CRC Press. Ch.5

Eriksson, J. E., Dechat, T., Grin, B., Helfand, B., Mendez, M., Pallari, H., & Goldman, R. D. (2009). Introducing intermediate filaments : from discovery to disease. *The Journal of Clinical Investigations*. **119**, 1763–1771.

Read, A. F. & Allen, J. A. (2000). The Economics of Immunity. *Science*. **290**, 1104–1105.

Falany, M. L., Thames 3rd, A. M., McDonald, J. M., Blair, H. C., McKenna, M. A., Moore, R. E., Young, M. K., & Williams, J. P. (2001). Osteoclasts secrete the chemotactic cytokine mim-1. *Biochem Biophys Res Commun*. **281**, 180–5.

Fields, S. (2001). Proteomics in Genomeland. *Science*, *291*, 1221–1224.

Firouzi, S., Studies, A., & Scien, B. (2014). Acute phase responses in commercial broiler chickens experimentally infected with a highly virulent Newcastle disease virus strain. *Online Journal of Veterinary Research*. **18**, 0–7.

Floris, G., Medda, R., Padiglia, A., & Musci, G. (2000). The physiopathological significance of ceruloplasmin. *Biochemical Pharmacology*. **60**, 1735–1741.

Fournier, M. L., Gilmore, J. M., Martin-Brown, S. A., & Washburn, M. P. (2007). Multidimensional Separations-Based Shotgun Proteomics. *Chemical Reviews*, **107**, 3654–3686.

Foutz, T., Ratterman, A., & Halper, J. (2007). Effects of Immobilization on the Biomechanical Properties of the Broiler Tibia and Gastrocnemius Tendon, *Poultry Science*. ***86***, 931–936.

Franceschini, G. (2003). Apolipoprotein function in health and disease: insights from natural mutations. *European Journal of Clinical Investigation*. **26**, 733–746.

Friedman, D. B., & Lilley, K. S. (2008). Optimizing the difference gel electrophoresis (DIGE) technology. *Methods in Molecular Biology*. **428**, 93–124.

Fujimoto, K., Cardelli, J., & Tso, P. (1992). Increased apolipoprotein A-IV in rat mesenteric lymph after lipid meal acts as a physiological signal for satiation. *American Journal of Physiology. Gastrointestinal and Liver Physiology*. **262**, G1002 – G1006.

Fujimoto, K., & Fukagawa, K. (1993). Suppression of food intake by apolipoprotein A-IV is mediated through the central nervous system in rats. *Journal of Clinical Investigation*. **91**, 1830–1833.

Fusek, M., & Vetvicka, V. (2005). Dual role of cathepsin D: ligand and protease. *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*. **149**, 43–50.

Gangneux, C., Daveau, M., Hiron, M., Derambure, C., Papaconstantinou, J., & Salier, J. P. (2003). The inflammation-induced down-regulation of plasma Fetuin-A (alpha2HS-Glycoprotein) in liver results from the loss of interaction between long C/EBP isoforms at two neighbouring binding sites. *Nucleic Acids Research*. **31**, 5957–70.

Garcia, K. O., Berchieri-Junior, A., Santana, A. M., Freitas-Neto, O. C., & Fagliari, J. J. (2009). Experimental infection of commercial layers using a Salmonella enterica serovar Gallinarum

strain: Leukogram and serum acute-phase protein concentrations. *Brazilian Journal of Poultry Science*. **11**, 263–270.

Georgieva, T. M., Koinarski, V. N., Urumova, V. S., Marutsov, P. D., Christov, T. T., Nikolov, J., Chaprazov, T., Walshe, K., Karov, R. S., Georgiev, I. P., & Koinarski, Z. V. (2010). Effects of Escherichia coli infection and Eimeria tenella invasion on blood concentrations of some positive acute phase proteins (haptoglobin (PIT 54), fibrinogen and ceruloplasmin). *Revue de Medecine Veterinaire*. **161,** 84–89.

Gharaibeh, S., & Mahmoud, K. (2013). Decay of maternal antibodies in broiler chickens. *Poultry Science*. **92**, 2333–6.

Giansanti, F., Giardi, M. F., & Botti, D. (2006). Avian Cytokines - An Overview. *Current Pharmaceutical Design*. **12**, 3083–3099.

Giansanti, F., Giardi, M. F., Massucci, M. T., Botti, D., & Antonini, G. (2007). Ovotransferrin expression and release by chicken cell lines infected with Marek's disease virus. *Biochemistry and Cell Biology*. **85**, 150–155.

Gilbert, E. R., Cox, C. . M., Williams, P. M., McElroy, A. P., Dalloul, R. A., Ray, W. K., Barri, A., Emmerson, D., Wong, E., & Webb, K. E. (2011). Eimeria species and genetic background influence the serum protein profile of broilers with coccidiosis. *PloS One*. ***6***, e14636.

Gilbert, E., Williams, P., & Ray, W. (2010). Proteomic evaluation of chicken brush-border membrane during the early posthatch period. *Journnal of Proteomic Research*. **9**, 4628–4639.

Goldfarb, V., Trimble, R. B., Falco, M., Liem, H. H., Metcalfe, S. A., Wellner, D., & Muller-Eberhard, U. (1986). An Avian Serum a-Glycoprotein , Hemopexin , Differing Significantly in both Amino Acid and Carbohydrate Composition from Mammalian (b-Glycoprotein) Counterparts. *Biochemistry*. **25**, 6555–6562.

Gordon, J. A, & Jencks, W. P. (1963). The relationship of structure to the effectiveness of denaturing agents for proteins. *Biochemistry*. **2**, 47–57.

Grieninger, G., Liang, T. J., Beuving, G., Goldfarb, V., Metcalfe, S. a, & Muller-Eberhard, U. (1986). Hemopexin is a developmentally regulated, acute-phase plasma protein in the chicken. *The Journal of Biological Chemistry*. **261**, 15719–24.

Grover, A., Shandilya, A., Agrawal, V., Pratik, P., Bhasme, D., Bisaria, V. S., & Sundar, D. (2011). Hsp90/Cdc37 chaperone/co-chaperone complex, a novel junction anticancer target elucidated by the mode of action of herbal drug Withaferin A. *BMC Bioinformatics*. **12,** *Suppl* S30.

Gruys, E., Toussaint, M. J. M., Niewold, T. A, & Koopmans, S. J. (2005). Acute phase reaction and acute phase proteins. *Journal of Zhejiang University. Science*. **6**, 1045–56.

Gruys, E., Toussaint, M. J. M., Upragarin, N., Van, E. A. M., Adewuyi, A. A., Candiani, D., Nguyen, T. K. A., & Sabeckiene, J. (2005). Acute phase reactants, challenge in the near future of animal production and veterinary medicine. *Journal of Zhejiang University. Science*. **6**, 941–947.

Gu, X. H., Hao, Y., & Wang, X. L. (2012). Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. *Poultry Science*. **91**, 790–9.

Guo, J. T., Aldrich, C. E., Mason, W. S., & Pugh, J. C. (1996). Characterization of serum amyloid A protein mRNA expression and secondary amyloidosis in the domestic duck. *Proceedings of the National Academy of Sciences of the United States of America*. **93**, 14548–53.

Yamanishi, H., Iyama S. & Yamaguchi Y. (2002). Modification of fully automated total iron-binding capacity (TIBC) assay in serum and comparison with dimension TIBC method. *Clinical Chemistry*. **48**, 1565–1570.

Hallquist, N. A., & Klasing, K. C. (1994). Serotransferrin, ovotransferrin and metallothionein levels during an immune response in chickens. *Comparative Biochemistry and Physiology. Biochemistry and Molecular Biology*. **108**, 375–84.

Hammond, D. M., & Long, P. L., (1973). *The Coccidia: Eimeria, Isospora, Toxoplasma, and Related Genera. Baltimore, MD. University Park Press.*

Hee, C. S., Fabian, H., Uchanska-Ziegler, B., Ziegler, A., & Loll, B. (2012). Comparative

biophysical characterization of chicken beta(2)-microglobulin. *Biophysical Chemistry*. **167**, 26–35.

Heegaard, P. M. H., Stockmarr, A., Pĩeiro, M., Carpintero, R., Lampreave, F., Campbell, F. M., Eckersall, P., Toussaint, M. J. M., Gruys, E., & Sorensen, N., (2011). Optimal combinations of acute phase proteins for detecting infectious disease in pigs. *Veterinary Research*. **42,** 50-63.

Henrion, D., Terzi, F., Matrougui, K., Duriez, M., Boulanger, C. M., Colucci-Guyon, E., Babinet, C., Briand, P., Friedlander, G., Poitevin, P., & Levy, B. I. (1997). Impaired flow-induced dilation in mesenteric resistance arteries from mice lacking vimentin. *The Journal of Clinical Investigation*. **100**, 2909–2914.

Hrubec, T., Whichard, J., Larsen, C., & Pierson, M. (2002). Plasma versus serum: specific differences in biochemical analyte values. *Journal of Avian Medicine and Surgery*. **16**, 101–105.

Hu, L., Ye, M., Jiang, X., Feng, S., & Zou, H. (2007). Advances in hyphenated analytical techniques for shotgun proteome and peptidome analysis-a review. *Analytica Chimica Acta*. **598**, 193–204.

Huang J, Tang X, Ruan J, Ma H, Z. S. (2010). Use of Comparative Proteomics to Identify Key Proteins Related to Hepatic Lipid Metabolism in Broiler Chickens: Evidence Accounting for Differential Fat Deposition Between Strains. *Lipids*. **45**, 81–85.

Ingenbleek, Y., & Bernstein, L. H. (2015). Plasma Transthyretin as a Biomarker of Lean Body Mass and Catabolic States. *Advances in Nutrition*. **6**, 572–580.

Ingenbleek, Y., Young, V. (1994). Transthyretin (Prealbumin) in Health and Disease: Nutritional Implications. *Annual Review of Nutrition*. **14**, 495–533.

Inoue, M., Satoh, W., & Murakami, H. (1997). Plasma alpha 1-acid glycoprotein in chickens infected with infectious bursal disease virus. *Avian Diseases*. **41,** 164–70.

Iseri, V. J., & Klasing, K. C. (2013). Dynamics of the systemic components of the chicken (Gallus gallus domesticus) immune system following activation by Escherichia coli; implications for the costs of immunity. *Developmental and Comparative Immunology*. **40**, 3-4

Issaq, H. J., & Veenstra, T. D. (2008). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): Advances and perspectives. *BioTechniques*. **44**,

Iwasaki, K., Morimatsu, M., Inanami, O., Uchida, E., Syuto, B., Kuwabara, M., & Niiyama, M. (2001). Isolation, characterization, and cDNA cloning of chicken turpentine-induced protein, a new member of the scavenger receptor cysteine-rich (SRCR) family of proteins. *The Journal of Biological Chemistry*. **276**, 9400–5.

Jawor, P., Steiner, S., Stefaniak, T., Baumgartner, W., & Rzasa, A. (2008). Determination of selected acute phase proteins during the treatment of limb diseases in dairy cows. *Veterinarni Medicinia*. **53**, 173–183.

Jielile, J., Jialili, A., Sabirhazi, G., Shawutali, N., Redati, D., Chen, J., Tang, B., Bai, J., & Aldyarhan, K. (2011). Proteomic analysis of differential protein expression of achilles tendon in a rabbit model by two-dimensional polyacrylamide gel electrophoresis at 21 days postoperation. *Applied Biochemistry and Biotechnology*. **165**, 1092–106.

Jones, R., Jordan, T., & Lioupis, S. (1975). Characteristics of reovirus isolated from ruptured gastrocnemius tendons of chickens. *Veterinary Record*. **96,** 153–154.

Juul-Madsen, H. R., Munch, M., Handberg, K. J., Sørensen, P., Johnson, A. A., Norup, L. R., & Jørgensen, P. H. (2003). Serum levels of mannan-binding lectin in chickens prior to and during experimental infection with avian infectious bronchitis virus. *Poultry Science*. **82**, 235–41.

Juul-Madsen, H. R., Norup, L. R., Handberg, K. J., & Jørgensen, P. H. (2007). Mannan-binding lectin (MBL) serum concentration in relation to propagation of infectious bronchitis virus (IBV) in chickens. *Viral Immunology*. **20**, 562–70.

Dziegielewska, K.M., Brown, W.M., Gould, C.C., Matthews, N., Sedgwick, J.E.C., and Saunders, N. R., (1992). Fetuin: an acute phase protein in cattle. *Journal of Comparative Physiology B. Biochemical*, Systems, and *Environmental Physiology*. **162**, 168–171.

Khovidhunkit, W., Duchateau, P. N., Medzihradszky, K. F., Moser, A. H., Naya-Vigne, J.,

Shigenaga, J. K., Kane, J. P., Grunfeld, C., Feingold, K. R., (2004). Apolipoproteins A-IV and A-V are acute-phase proteins in mouse HDL. *Atherosclerosis*. **176**, 37–44.

Khurana, S., & George, S. P. (2008). Regulation of cell structure and function by actin-binding proteins: Villin's perspective. *FEBS Letters*, **582**, 2128–2139.

Kibenge, F. S. B., Wilcox, G. E., & Pass, D. A. (1983). Pathogenicity of four strains of staphylococci isolated from chickens with clinical tenosynovitis. *Avian Pathology*. **12**, 213–220.

King, S. A., & McLelland, J. (1984). *Birds: Their structure and Function*. Bath, Bailliere Tindall.

Klasing, K. (2004). The costs of immunity. *Acta Zoologica Sinica*. **50**, 961–969.

Kogut, M. H. (2009). Impact of nutrition on the innate immune response to infection in poultry. *Journal of Applied Poultry Research*. **18**, 111–124.

Koh, T. S., Peng, R. K., & Klasing, K. C. (1996). Dietary copper level affects copper metabolism during lipopolysaccharide-induced immunological stress in chicks. *Poultry Science*. **75**, 867–72.

Korte, J., Fröhlich, T., Kohn, M., Kaspers, B., Arnold, G. J., & Härtle, S. (2013). 2D DIGE analysis of the bursa of Fabricius reveals characteristic proteome profiles for different stages of chicken B-cell development. *Proteomics*. **13**, 119–133.

Kravitz, M. S., Pitashny, M., & Shoenfeld, Y. (2005). Protective molecules--C-reactive protein (CRP), serum amyloid P (SAP), pentraxin3 (PTX3), mannose-binding lectin (MBL), and apolipoprotein A1 (Apo A1), and their autoantibodies: prevalence and clinical significance in autoimmunity. *Journal of Clinical Immunology*. **25**, 582–91.

Labas, V., Grasseau, I., Cahier, K., Gargaros, A., Harichaux, G., Teixeira-Gomes, A. P., Alves, S., Bourin, M., Gerard, N., & Blesbois, E. (2015). Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *Journal of Proteomics*. **112**, 313–335.

Labat-Robert, J. & Robert, L., (2012). Fifty years of structural glycoproteins. *Pathologie-biologie,* **60**: 66–75.

Laemmli, U. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**, 680 – 686.

Lamon-Fava, S., Sastry, R., Ferrari, S., Rajavashisth, T. B., Lusis, a J., & Karathanasis, S. K. (1992). Evolutionary distinct mechanisms regulate apolipoprotein A-I gene expression: differences between avian and mammalian apoA-I gene transcription control regions. *Journal of Lipid Research*. **33**, 831–42.

Landman, W., Gruys, E., & Dwars, R. (1994). A syndrome associated with growth depression and amyloid arthropathy in layers: a preliminary report. *Avian Pathology*. **23**, 461–470.

Landman, W. J. M. (1998). *Amyloid Arthropathy*. Ph.D. Utrecht University.

Lange, V., Picotti, P., Domon, B., & Aebersold, R. (2008). Selected reaction monitoring for quantitative proteomics: a tutorial. *Molecular Systems Biology*, *4. Article* 222.

Lassaletta, L., Billen, G., Grizzetti, B., Garnier, J., Leach, A. M., & Galloway, J. N. (2014). Food and feed trade as a driver in the global nitrogen cycle: 50-year trends. *Biogeochemistry*. **118**, 225–241.

Laursen, S. B., & Nielsen, O. L. (2000). Mannan-binding lectin (MBL) in chickens: molecular and functional aspects. *Developmental and Comparative Immunology*. **24**, 85–101.

Laursen, S., Hedemand, J., Nielsen, O., Thiel, S., Koch, C., & Jensenius, J. C. (1998). Serum levels, ontogeny and heritability of chicken mannan-binding lectin (MBL). *Immunology*. **94**, 587–593.

Lawless, C., & Hubbard, S. J. (2012). Prediction of missed proteolytic cleavages for the selection of surrogate peptides for quantitative proteomics. *Omics : A Journal of Integrative Biology*. **16**, 449–56.

Lee, K. W., Lillehoj, H. S., Lee, S. H., Jang, S. I., Ritter, G. D., Bautista, D. A., & Lillehoj, E. P. (2011). Impact of Fresh or Used Litter on the Posthatch Immune System of Commercial Broilers Impact of Fresh or Used Litter on the Posthatch Immune System of Commercial Broilers. *Avian Diseases*. **55**, 539–544.

Lequin, R. M. (2005). Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical Chemistry*. **51**, 2415–2418.

Lewis, E. J., & Bishop J, A. S., (1998). A simple inflammation model that distinguishes between

the actions of anti-inflammatory and anti-rheumatic drugs. *Inflammation Research*. **47**, 26–35.

Li, G. H., Arora, P. A., Chen, Y., McCulloch, C. A., & Liu, P., (2012). Multifunctional Roles of Gelsolin in Health and Diseases. *Medicinal Research Reviews*. **32**, 999–1025.

Lilley, K. S., & Friedman, D. B. (2004). All about DIGE: quantification technology for differential-display 2D-gel proteomics. *Expert Review of Proteomics*. **1**, 401 – 409.

Little, D., Thompson, J. W., Dubois, L. G., Ruch, D. S., Moseley, M. A., & Guilak, F. (2014). Proteomic Differences between Male and Female Anterior Cruciate Ligament and Patellar Tendon. *PloS One*, *9*(5), e96526. doi:10.1371/journal.pone.0096526

Liu, L., Shen, J., Zhao, C., Wang, X., Yao, J., Gong, Y., & Yang, X. (2015). Dietary Astragalus polysaccharide alleviated immunological stress in broilers exposed to lipopolysaccharide. *International Journal of Biological Macromolecules*. **72**, 624–632.

Luo, J., Zheng, A., Meng, K., Chang, W., Bai, Y., Li, K., Cai, H., Liu, G.A., & Yao, B. (2013). Proteome changes in the intestinal mucosa of broiler (Gallus gallus) activated by probiotic Enterococcus faecium. *Journal of Proteomics*. **91**, 226–41.

Lynagh, G. R., Collins, R. A, & Kaiser, P. (2000). Development and use of monoclonal antibodies to chicken fibronectin to show that the chicken hepatocellular carcinoma cell line, LMH, constitutively expresses fibronectin. *Research in Veterinary Science*. **68**, 147–52.

Lynch, N. J., Khan, S., Cordula, M., Sandrini, S. M., Marston, D., Presanis, J. S., Schwaeble, W. J., & Stover, C. M., (2005). Composition of the Lectin Pathway of Complement in Gallus gallus: Absence of Mannan-Binding Lectin-Associated Serine Protease-1 in Birds. *Journal of Immunology*. **174**, 4998–5006.

Mancini, G., Carbonara, A. O., & Heremans, J. F. (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*. **2**, 235–254.

Martínez-Subiela, S., Tecles, F., & Ceron, J. J. (2007). Comparison of two automated spectrophotometric methods for ceruloplasmin measurement in pigs. *Research in Veterinary Science.*, **83**, 12–9.

Megger, D. A., Bracht, T., Meyer, H. E., & Sitek, B. (2013). Label-free quantification in clinical proteomics. *Biochimica et Biophysica Acta - Proteins and Proteomics*. **1834**, 1581–1590.

Mhawech, P. (2005). 14-3-3 Proteins-an Update. *Cell Research*. **15**, 228–36.

Mikšík, I., Ergang, P., & Pácha, J. (2014). Proteomic analysis of chicken eggshell cuticle membrane layer. *Analytical and Bioanalytical Chemistry*. *406*, 7633–7640.

Miller, I. (2011). Protein Separation Strategies. In Eckersall, P. D. & Whitfield P. D. ed, *Methods in Animal Proteomics*. Oxford UK, Wiley-Blackwell. Ch.1.

Millet, S., Bennett, J., Lee, K. A, Hau, M., & Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Developmental and Comparative Immunology*. **31**, 188–201.

Mireles, A. J., Kim, S. M., & Klasing, K. C. (2005). An Acute Inflammatory Response Alters Bone Homeostasis , Body Composition , and the Humoral Immune Response of Broiler Chickens. *Poultry Science*. ***84***, 553–560.

Moussa M, Swider P, Babilé R, Fernandez X, R. H. (2008). Effects of Physical Activities on Biochemical and Biomechanical Properties of Tendons in Two Commercial Types of Chickens. *Connective Tissue Research*, **49**, 76–84.

Murata, H., Shimada, N., & Yoshioka, M. (2004). Current research on acute phase proteins in veterinary diagnosis: an overview. *Veterinary Journal*. **168**, 28–40.

Musquera S, Lombart C, Jayle MF, Rogard M, Waks, M. (1979). Identification of haptoglobin in chicken serum and specificity of the chicken haptoglobin-hemoglobin complex formation. *Comparative Biochemistry* and *Physiology* Part *B*: *Biochemistry and Molecular Biology*, **62**, 241–244.

Nag, S., Larsson, M., Robinson, R. C., & Burtnick, L. D. (2013). Gelsolin: The tail of a molecular gymnast. *Cytoskeleton*. **70**, 360–384.

Najafi, P., Nurfarahin, a J., Soleimani, a F., Kumari, S., Aryani, a A., Reilly, E. L. O., & Eckersall, P. D. (2009). Acute phase proteins , interleukin 6 , and heat shock protein 70 in broiler chickens administered with corticosterone. *Poultry Science*, 3112–3118.

Najafi, P., Zulkifli, I., Amat Jajuli, N., Farjam, A. S., Ramiah, S. K., Amir, A. A., … Eckersall, D. (2015). Environmental temperature and stocking density effects on acute phase proteins, heat shock protein 70, circulating corticosterone and performance in broiler chickens. *International Journal of Biometeorology*. **59**:1577-1583.

Najafi, P., Zulkifli, I., Soleimani, A. F., & Kashiani, P. (2015). The effect of different degrees of feed restriction on heat shock protein 70 , acute phase proteins , and other blood parameters in female broiler breeders. *Poultry Science*. **94**, 2322–2329.

Nakamura, K., Imai, K., & Tanimura, N. (1996). Comparison of the effects of infectious bronchitis and infectious laryngotracheitis on the chicken respiratory tract. *Journal of Comparative Pathology*. **114**,11–21.

Nakamura, K., Mitarai, Y., & Yoshioka, M. (1998). Serum levels of interleukin-6, alpha1-acid glycoprotein, and corticosterone in two-week-old chickens inoculated with Escherichia coli lipopolysaccharide. *Poultry Science*. **77**, 908–911.

Navarro, M. A., Acin, S., Iturralde, M., Calleja, L., Carnicer, R., Guzmán-García, M. A., González-Ramón, N., Mata, P., Isabel, B., Lopez-Bote, C. J., Lampreave, F., Piñeiro, A., & Osada, J. (2004). Cloning, characterization and comparative analysis of pig plasma apolipoprotein A-IV. *Gene*. **325**, 157–164.

Navarro, M. A., Acin, S., Iturralde, M., Calleja, L., Carnicer, R., Surra, J. C., Guzmán-García, M. A., González-Ramón, N., Iturralde, M., Lampreave, F., Piñeiro, A., & Osada, J. (2005). Immune-regulation of the apolipoprotein A-I/C-III/A-IV gene cluster in experimental inflammation. *Cytokine*. **31**, 52–63.

Nazifi, S., Dadras, H., Hoseinian, S. a., Ansari-Lari, M., & Masoudian, M. (2010). Measuring acute phase proteins (haptoglobin, ceruloplasmin, serum amyloid A, and fibrinogen) in healthy and infectious bursal disease virus-infected chicks. *Comparative Clinical Pathology*. **19**, 283–286.

Nazifi, S., Tabande, M. R., Hosseinian, S. a., Ansari-Lari, M., & Safari, H. (2011). Evaluation of sialic acid and acute-phase proteins (haptoglobin and serum amyloids A) in healthy and avian infection bronchitis virus-infected chicks. *Comparative Clinical Pathology*. **20**, 69–73.

Neeteson, A. M. (2010). Striking the Balance. *Journal of Animal Breeding and Genetics*. **127**, 85–86.

Nesvizhskii, A. I. (2007). Protein Identification by Tandem Mass Spectrometry and Sequence Database Searching. In Matthiesen, R. Ed. *Mass Spectrometry Data Analysis in Proteomics*. Clifton, New Jersery, Humana Press.

Nicholas P. C. Horrocks, B. I. T. and K. D. M. (2011). A simple assay for measurement of ovotransferrin–a marker of inflammation and infection in birds. *Methods in Ecology and Evolution*. **11**, 518–526.

Nielsen, O. L., Jensenius, J. C., Jørgensen, P. H., & Laursen, S. B. (1999). Serum levels of chicken mannan-binding lectin (MBL) during virus infections; indication that chicken MBL is an acute phase reactant. *Veterinary Immunology and Immunopathology*. **70**, 309–16.

Nielsen, O. L., Jørgensen, P. H., Hedemand, J., Jensenius, J. C., Koch, C., & Laursen, S. B. (1998). Immunohistochemical investigation of the tissue distribution of mannan-binding lectin in non-infected and virus-infected chickens. *Immunology*. **94**, 122–8.

Nielsen, O. L., Sørensen, P., Hedemand, J. E., Laursen, S. B., & Jørgensen, P. H. (1998). Inflammatory response of different chicken lines and B haplotypes to infection with infectious bursal disease virus. *Avian Pathology*. **27**, 181–9.

Nogueria, F., & Domont, G. (2014). Survey of Shotgun proteomics. *Methods in Molecular Biology*. **1156**, 3–23.

O'Farrell, P. H. (1975). High Resolution of Proteins Electrophoresis. *The Journal of Biological Chemistry*. **250**, 4007–4021.

O'Reilly, E. L. (2013). *The effect of microbial challenge on the intestinal proteome of broiler chickens*. M.Sc. Univeristy of Glasgow.

O'Reilly, E. L., Eckersall, P. D. (2014). Acute phase proteins: a review of their function, behaviour and measurement in chickens. *World's Poultry Science Journal*. **70**, 27–44.

Oikawa, S., Katoh, N., Itoh, H., Miyamoto, T., Konno, M., & Kajita, T. (1997). Decreased serum

apolipoprotein A-I concentrations in cows infected with Salmonella typhimurium. *Canadian Journal of Veterinary Research, Revue Canadienne de Recherche Vétérinaire*. **61**, 182–6.

Olivier, E., Soury, E., Ruminy, P., Husson, A., Parmentier, F., Daveau, M., & Salier, J. P. (2000). Fetuin-B, a second member of the fetuin family in mammals. *The Biochemical Journal*. **350**, 589–97.

Önnerfjord, P., Khabut, A., Reinholt, F. P., Svensson, O., & Heinegård, D. (2012). Quantitative proteomic analysis of eight cartilaginous tissues reveals characteristic differences as well as similarities between subgroups. *The Journal of Biological Chemistry*. **287**, 18913–24.

Oravainen, J. (2008). *Field studies on infectious reproductive diseases and lameness in sows*. Ph.D. University of Helsinki.

Otaka, M., Odashima, M., Tamaki, K., & Watanabe, S. (2009). Expression and function of stress (heat shock) proteins in gastrointestinal tract. *International Journal of Hyperthermia*. **25**, 634–40.

Ouchterlony, O. (1949). Antigen- antibody reactions in gels. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*. ***26***, 507–515.

Ovelgönne, J. H., Landman, W. J., Gruys, E., Gielkens, a L., & Peeters, B. P. (2001). Identical amyloid precursor proteins in two breeds of chickens which differ in susceptibility to develop amyloid arthropathy. *Amyloid :The Official Journal of the International Society of Amyloidosis*. **8**, 41–51.

Pan, X., Munshi, M. K., Iqbal, J., Queiroz, J., Sirwi, A. A., Shah, S., … Hussain, M. M. (2013). Circadian regulation of intestinal lipid absorption by apolipoprotein AIV involves forkhead transcription factors A2 and O1 and microsomal triglyceride transfer protein. *Journal of Biological Chemistry*. **288**, 20464–20476.

Pancholi, V. (2001). Cellular and Molecular Life Sciences Multifunctional a -enolase : its role in diseases. Cellular and Molecular Life Sciences. **58**, 902–920.

Pardina, E., López-Tejero, M. D., Llamas, R., Catalán, R., Galard, R., Allende, H., Vargas, V., Lecube, A., Fort, J. M., Baena-Fustegueras, J. A., & Peinado-Onsurbe, J. (2009). Ghrelin and apolipoprotein AIV levels show opposite trends to leptin levels during weight loss in morbidly obese patients. *Obesity Surgery*. **19**, 1414–23.

Patterson, L. T., & Mora, E. C. (1964). Occurance of a substance analougus to C-Reactive protein in the blood of domestic fowl. *Texas Reports on Biology and Medicine*. **22**, 716–721.

Patterson, L. T., & Mora, E. C. (1965). The C-reactive protein response and disease resistance in domestic fowl. *Texas Reports on Biology and Medicine*. **23**, 600–606.

Peebles, E. D., Jacob, R., Branton, S. L., & Gerard, P. D. (2014). Effects of Mycoplasma gallisepticum vaccination on serum α1-acid glycoprotein concentrations in commercial layer chickens. *Poultry Science*. **93**, 1396–402.

Peters, S. M., Yancy, H., Deaver, C., Jones, Y. L., Kenyon, E., Chiesa, O. A., Esparza, J., Screven, R., Lancaster, V., Stubbs, J. T., Yang, M., Wiesenfeld, P. L., & Myers, M. J. (2012). In vivo characterization of inflammatory biomarkers in swine and the impact of flunixin meglumine administration. *Veterinary Immunology and Immunopathology*. **148**, 236–42.

Petersen, H. H., Nielsen, J. P., & Heedaard, P. M. H. (2004). Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary Research*. **35**, 163–187.

Pharmaceutica, J. (1990). *Diagnosis of Coccidiosis in Chickens*.

Piñeiro, M., Piñeiro, C., Carpintero, R., Morales, J., Campbell, F. M., Eckersall, P. D., Toussaint, M. J. M., & Lampreave, F. (2007). Characterisation of the pig acute phase protein response to road transport. *Veterinary Journal*. **173**, 669–74.

Poernama, F., Subramanian, R., Cook, M. E., & Attie, A. D. (1992). High density lipoprotein deficiency syndrome in chickens is not associated with an increased susceptibility to atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **12**, 601–607.

Qin, X., Swertfeger, D. K., Zheng, S., Hui, D. Y., Tso, P., Hui, Y., & Aiv, A. (1998). Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation. *American Journal of Physiology - Heart and Circulatory Physiology*. **274**, H1836–H1840.

Rajavashisth, T. B., Dawson, P. a, Williams, D. L., Shackleford, J. E., Lebherz, H., & Lusis, A. J.

(1987). Structure, evolution, and regulation of chicken apolipoprotein A-I. *The Journal of Biological Chemistry*. **262**, 7058–65.

Rasaputra, K. S., Liyanage, A. B. R., Lay, J. O., McCarthy, F. M., & Rath, N. C. (2010). Tibial dyschondroplasia-associated proteomic changes in chicken growth plate cartilage. *Avian Diseases*. **54**, 1166–1171.

Rath, N. C., Anthony, N. B., Kannan, L., Huff, W. E., Huff, G. R., Chapman, H. D., Erf, G. F., & Wakenell, P. (2009). Serum ovotransferrin as a biomarker of inflammatory diseases in chickens. *Poultry Science*. **88**, 2069–74.

Rauber, R. H., Oliveira, M. S., Mallmann, A. O., Dilkin, P., Mallmann, C. A., Giacomini, L. Z., & Nascimento, V. P. (2013). Effects of fumonisin B1 on selected biological responses and performance of broiler chickens. *Pesquisa Veterinaria Brasileira*. **33**, 1081–1086.

Rauber, R. H., Perlin, V. J., Fin, C. D., Mallmann, A. L., Miranda, D. P., Giacomini, L. Z., &
Nascimento, V. P. (2014). Interference of Salmonella typhimurium Lipopolysaccharide on Performance and Biological Parameters of Broiler Chickens. *Brazilian Journal of Poultry Science*. **16,** *67 -76*

Richards, M. P., & Augustine, P. C. (1988). Serum and liver zinc, copper, and iron in chicks infected with Eimeria acervulina or Eimeria tenella. *Biological Trace Element Research*. **17**, 207–19.

Riddell, C. (1983). Pathology of the skeleton and tendons of broiler chickens reared to roaster weights - Crippled chickens. *Avian Diseases*. **87**, 950–962.

Röcken, C., & Shakespeare, A. (2002). Pathology, diagnosis and pathogenesis of AA amyloidosis. *Virchows Archiv*. **440**, 111–122.

Rodriguez, M. D., Kalogeris, T. J., Wang, X. L., Wolf, R. T. P. (1997). Rapid synthesis and secretion of intestinal apolipoprotein A-IV after gastric fat loading in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. **272**, R1170–R1177.

Roman, Y., Bed'hom, B., Guillot, A., Levrier, J., Chaste-Duvernoy, D., Bomsel-Demontoy, M.-C., & Jalme, M. S. (2009). Identification of apolipoprotein A-I in the alpha-globulin fraction of avian plasma. *Veterinary Clinical Pathology / American Society for Veterinary Clinical Pathology*. **38**, 206–12.

Roy, K., Kjelgaard-Hansen, M., Pors, S. E., Christensen, J. P., Biswas, P. K., & Bojesen, A. M. (2014). Performance of a commercial Chicken-Ovo-transferrin-ELISA on the serum of brown layer chickens infected with Gallibacterium anatis and Streptococcus zooepidemicus. *Avian Pathology*. **43**, 57–61.

Sadeyen, J.-R., Trotereau, J., Protais, J., Beaumont, C., Sellier, N., Salvat, G., Velge, P., & Lalmanach, A.-C. (2006). Salmonella carrier-state in hens: study of host resistance by a gene expression approach. *Microbes and Infection / Institut Pasteur*. **8**, 1308–14.

Salamano, G., Mellia, E., Tarantola, M., Gennero, M. S., Doglione, L., & Schiavone, a. (2010). Acute phase proteins and heterophil:lymphocyte ratio in laying hens in different housing systems. *The Veterinary Record*. **167**, 749–51.

Saleem, G. (2013). Necrotic Enteritis , disease induction , predisposing factors and novel biochemical markers in broiler chickens. Ph.D. *University of Glasgow*.

Salvidor, A., & Levin, Y. (2014). Quantification of Proteins by Label-Free LC-MS. *Methods in Molecular Biology*. ***1156***, 223–247.

Santiago-Cardona, P. G., Berríos, C. A., Ramírez, F., & García-Arrarás, J. E. (2003). Lipopolysaccharides induce intestinal serum amyloid A expression in the sea cucumber Holothuria glaberrima. *Developmental & Comparative Immunology*. **27**, 105–110.

Sato, T., Nakamoto, A., Hori, N., Enoki, Y., Fukushima, Y., Nakamoto, N., Sakata, Y., Yamanaka, H., Chida, D., Abe, T. and Yoda, T. (2012). proteomic analysis of masticatory muscle tendon–aponeurosis hyperplasia: A preliminary study using a 2D-DIGE system. *Journal of Oral and Maxillofacial Surgery, Medicine, and Pathology*. **24**, 185–188.

Schou, T. W., Permin, a, Christensen, J. P., Cu, H. P., & Juul-Madsen, H. R. (2010). Mannan-binding lectin (MBL) in two chicken breeds and the correlation with experimental Pasteurella multocida infection. *Comparative Immunology, Microbiology and Infectious*

Diseases. **33**,183–95.

Seifi, S., H, A. S. S., & K, A. F. (2014). Acute phase response in experimentally infected broilers with avian infectious bronchitis virus serotype 4/91. *Journal of the Hellenic Veterinary Medical Society*. **65**, 17–22.

Sellers, J. R. (2000). Myosins: a diverse superfamily. *Biochimica et Biophysica Acta*. **1496**, 3–22.

Sentandreu, M. A., Fraser, P. D., Halket, J., Patel, R., & Bramley, P. M. (2010). A proteomic based approach for detection of chicken in meat mixes. *Journal of Proteome Research*,. ***9***, 3374–3383.

Sevimli, A., Misirlioğlu, D., Polat, U., Yalçin, M., Akkoç, A., & Uğuz, C. (2005). The effects of vitamin A, pentoxyfylline and methylprednisolone on experimentally induced amyloid arthropathy in brown layer chicks. *Avian Pathology*. **34**, 143–9.

Shahzad, M., Gao, J., Qin, P., Liu, J., Wang, Z., Zhang, D., & Li, J. (2014). Expression of Genes encoding matrilin-3 and cyclin-I during the impairment and recovery of chicken growth plate in tibial dyschondroplasia. *Avian Diseases*. **58**, 468–73.

Shainkin-Kestenbaum, R., Berlyne, G., Zimlichman, S., Sorin, H. R., Nyska, M., & Danon, a. (1991). Acute phase protein, serum amyloid A, inhibits IL-1- and TNF-induced fever and hypothalamic PGE2 in mice. *Scandinavian Journal of Immunology*. **34**, 179–83.

Shakeri, M., Zulkifli, I., Soleimani, A. F., OReilly, E. L., Eckersall, P. D., Aryani, A. A., Kumari, S. & Abdullah, F. F. J. (2014). Response to dietary supplementation of L -glutamine and L -glutamate in broiler chickens reared at different stocking densities under hot , humid tropical conditions. *Poultry Science*. **93**, 2700–2708.

Simon, T., Cook, V. R., Rao, A., & Weinberg, R. B. (2011). Impact of murine intestinal apolipoprotein A-IV expression on regional lipid absorption, gene expression, and growth. *The Journal of Lipid Research*. **52**, 1984–1994.

Sitar, M.E., Aydin, S. & Cakatay, U., (2013). Human serum albumin and its relation with oxidative stress. *Clinical laboratory*, **59**: 945–52.

Sloop, C. H., Dory, L., Krause, B. R., Castle, C., & Roheim, P. S. (1983). Lipoproteins and apolipoproteins in peripheral lymph of normal and cholesterol-fed dogs. *Atherosclerosis*, **49**, 9–21.

Sohail, M. U., Ijaz, A, Yousaf, M. S., Ashraf, K., Zaneb, H., Aleem, M., & Rehman, H. (2010). Alleviation of cyclic heat stress in broilers by dietary supplementation of mannan-oligosaccharide and Lactobacillus-based probiotic: dynamics of cortisol, thyroid hormones, cholesterol, C-reactive protein, and humoral immunity. *Poultry Science*. **89**, 1934–8.

Sokale, A., Peebles, E. D., Zhai, W., Pendarvis, K., Burgess, S., & Pechan, T. (2012). Proteome profile of the pipping muscle in broiler embryos. *Proteomics*. **11**, 4262–4265.

Soler L, Molenaar A, Merola N, Eckersall PD, Gutiérrez A, Cerón JJ, Mulero V, N. T. (2013). Why working with porcine circulating serum amyloid A is a pig of a job. *Journal of Theoretical Biology*. **317**, 119–125.

Song, Z., Zhu, L., Zhao, T., Jiao, H., & Lin, H. (2009). Effect of copper on plasma ceruloplasmin and antioxidant ability in broiler chickens challenged by lipopolysaccharide. *Asian-Australasian Journal of Animal Sciences*. **22**, 1400–1406.

Spring, J., Beck, K., & Chiquet-Ehrismann, R. (1989). Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell.*, **59**, 325–34.

Stavitsky, A. B. (2007). The innate immune response to infection, toxins and trauma evolved into networks of interactive, defensive, reparative, regulatory, injurious and pathogenic pathways. *Molecular Immunology*. **44**, 2787–2799.

Steelman, S. M., & Chowdhary, B. P. (2012). Plasma proteomics shows an elevation of the anti-inflammatory protein APOA-IV in chronic equine laminitis. *BMC Veterinary Research*. **8**, 179-193

Steinmetz, A., Hermann, M., Nimpf, J., Aebersold, R., Ducret, A., Weinberg, R. B., & Schneider, W. J. (1998). Expression and conservation of apolipoprotein AIV in an avian species. *The Journal of Biological Chemistry*. **273**, 10543–9.

Steinmetz, A. & Schneider, W. J. (1999). Evolving functions of apolipoprotein AIV: from birds to mammals. *Trends in Cardiovascular Medicine*. **9**, 153–7.

Stevens, F. J. (2004). Hypothetical structure of human serum amyloid A protein. *Amyloid*. **11**, 71–80.

Stevens, L. (1991). Egg white proteins. *Comparative iochemistry and Physiology Part B: Biochemistry and Molecular Biology*. **100**, 1-9.

Sunde, M., Richardson, S. J., Chang, L., Pettersson, T. M., Schreiber, G., & Blake, C. C. (1996). The crystal structure of transthyretin from chicken. *European Journal of Biochemistry/FEBS*, **236**, 491–499.

Sunderman, F., & Nomoto, S. (1970). Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. *Clinical Chemistry*. **16**, 903–910.

Swaggerty, C., Genovese, K., & He, H. (2011). Broiler breeders with an efficient innate immune response are more resistant to Eimeria tenella. *Poultry Science*. **90**, 1014–9.

Swaggerty, C. L., Kaiser, P., Rothwell, L., Pevzner, I. Y., & Kogut, M. H. (2006). Heterophil cytokine mRNA profiles from genetically distinct lines of chickens with differential heterophil-mediated innate immune responses. *Avian Pathology*. **35**, 102–108.

Swaggerty, C. L., Pevzner, I. Y., He, H., Genovese, K. J., Nisbet, D. J., Kaiser, P., & Kogut, M. H. (2009). Selection of broilers with improved innate immune responsiveness to reduce on-farm infection by foodborne pathogens. *Foodborne Pathogens and Disease*. **6**, 777–783.

Sylte, M. J., & Suarez, D. L. (2012). Vaccination and acute phase mediator production in chickens challenged with low pathogenic avian influenza virus; novel markers for vaccine efficacy? *Vaccine*. **30**, 3097–105.

Takahashi, K., Kaji, N., Akiba, Y., & Tamura, K. (1994). Plasma alpha 1-acid glycoprotein concentration in broilers: influence of age, sex and injection of Escherichia coli lipopolysaccharide. *British Poultry Science*. **35**, 427–32.

Takahashi, K., Miyake, N., & Ohta, T. (1998). Changes in plasma alpha 1-acid glycoprotein concentration and selected immune response in broiler chickens injected with Escherichia coli lipopolysaccharide. *British Poultry Science*. **39**, 152 –155.

Takahashi, K., Takagi, K., & Akiba, Y. (2009). Effects of dietary glycine supplementation and fish meal on inflammatory responses in broiler chicks. *British Poultry Science*. **50**, 479–86.

Takahashi, K., Yodogawa, S., Akiba, Y., & Tamura, K. (1995). Effect of dietary protein concentration on responses to Escherichia coli endotoxin in broiler chickens. *British Journal of Nutrition*. **74**, 173 – 182.

Takahashi, Y., Konishi, H., Sato, K., Oohashi, T., & Miyamoto, T. (2004). Enzyme-linked immunosorbent assay for bovine apolipoprotein A-IV. *The Journal of Veterinary Medical Science/the Japanese Society of Veterinary Science*. **66**, 1199–204.

Takahashi, K., Akiba, Y., Iwata, T. and Kasai, M. (2002). Dietary conjugated linoleic acids alleviate early inflammatory response caused by lipopolysaccharide injection in male broiler chicks. *Animal Science Journal*. **73**, 47–50.

Tan, B., Foo, H., Loh, T., Norhani, A., & Zulkifli, I. (2005). Purification and characterization of very low density lipoprotein in commercial broiler and crossbred village chickens by fast protein liquid chromatography. Asian-Australasian Journal of Animal Sciences. **18**, 1780–1785.

Tang, X., Meng, Q., Gao, J., Zhang, S., & Zhang, H. (2015). Label-free Quantitative Analysis of Changes in Broiler Liver Proteins under Heat Stress using SWATH-MS Technology. *Nature Publishing Group*. **5**, 1–15.

Thanthrige-Don, N., Parvizi, P., Sarson, A. J., Shack, L. a, Burgess, S. C., & Sharif, S. (2010). Proteomic analysis of host responses to Marek's disease virus infection in spleens of genetically resistant and susceptible chickens. *Developmental and Comparative Immunology*. **34**, 699–704.

Thibodeau, S. N., Lee, D. C., & Palmiter, D. (1978). Identical precursors for serum transferrin add egg white conalbumin. The Journal of Biological Chemistry. **253**, 3771–3774.

Thrall, M., Baker, D., Campbell, T. W., DeNicola, D., Fettman, M., Lassen, E., … Weiser, G. (2004). *Veterinary Hematology and Clinical Chemistry*. John Wiley & Sons.

Tidball, J. G. (1992). Desmin at myotendinous junctions. *Experimental Cell Research*. **199**, 206–12.

Tillett, W., & Francis, T. (1930). Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *The Journal of Experimental Medicine*. **52**, 561–571.

Tolosano, E., & Altruda, F. (2002). Hemopexin: structure, function, and regulation. *DNA and Cell Biology*. **21**, 297–306.

Tolosano, E., Fagoonee, S., Morello, N., Vinchi, F., & Fiorito, V. (2010). Heme scavenging and the other facets of hemopexin. *Antioxidants & Redox Signaling*. **12,** 305–20.

Torok, V. a, Hughes, R. J., Ophel-Keller, K., Ali, M., & Macalpine, R. (2009). Influence of different litter materials on cecal microbiota colonization in broiler chickens. *Poultry Science*. **88**, 2474–81.

Tso, P., & Liu, M. (2004). Apolipoprotein A-IV, food intake, and obesity. *Physiology and Behavior*. **83**, 631–643.

Tso, P., Liu, M., & Kalogeris, T. (1999). The role of apolipoprotein A-IV in food intake regulation. *The Journal of Nutrition*. **129**, 1503–1506.

Tugendreich, S., Pearson, C., Sagartz, J., Jarnagin, K., & Kolaja, K. (2006). NSAID-Induced Acute Phase Response is Due to Increased Intestinal Permeability and Characterized by Early and Consistent Alterations in Hepatic Gene Expression. *Toxicologic Pathology*. **34**, 168–179.

Tuyttens, F., Heyndrickx, M., De Boeck, M., Moreels, A., Van Nuffel, A., Van Poucke, E., Van Coillie, E., Van Dongen, S., & Lens, L. (2008). Broiler chicken health, welfare and fluctuating asymmetry in organic versus conventional production systems. *Livestock Science*. **113**, 123–132.

Uhlar, C. M., & Whitehead, A. S., (1999). Serum amyloid A, the major vertebrate acute-phase reactant. *European Journal of Biochemistry/FEBS*, **265**, 501–23.

Uhrin, P., Perkmann, T., Binder, B., & Schabbauer, G. (2013). ISG12 is a critical modulator of innate immune responses in murine models of sepsis. *Immunobiology*. **218**, 1207–16.

Unlu, M., & Minden, J. (2002). Difference gel electrophoresis. In Walker J. Ed. *The protein protocols handbook* (2nd ed).Humana Press

Unlu, M., Morgan, M. E., & Minden, J. S. (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis*. **18**, 2071–7.

Upragarin, N. (2005). *In vitro studies on the pathogenesis of AA amyloid arthropathy in chicken*. Ph.D. Utrecht University.

Upragarin, N., Asten, A. J. a M. Van, J Tooten, P. C., M Landman, W. J., & Gruys, E. (2005). Serum amyloid A production by chicken fibroblast-like synoviocytes. *Veterinary Immunology and Immunopathology*. **106**, 39–51.

Durairaj, V., Okimoto, R., Rasaputra, K., Clark, F. D., & Rath, N. C. R. (2009). Histopathology and serum clinical chemistry evaluation of broilers with femoral head separation disorder. *Avian Diseases,*. **53**, 21–25.

Van Droogenbroeck, C., Beeckman, D. S. a, Harkinezhad, T., Cox, E., & Vanrompay, D. (2008). Evaluation of the prophylactic use of ovotransferrin against chlamydiosis in SPF turkeys. *Veterinary Microbiology*.**132**, 372–8.

van Walsum, J., Vertommen, M. H., & v.d. Laan A. (1981). Contribution to the aetiology of synovitis in chickens, with special reference to non-infective factors. V. *Veterinary Quarterly*. **3,** 111–117.

Van Weemen, B. K., & Schuurs, A. H. W. M. (1971). Immunoassay using antigen-enzyme conjugates. *FEBS Letters*. **15**, 232–236.

Vermeulen, A., Schaap, D., & Schetters, T. (2001). Control of coccidiosis in chickens by vaccination. *Veterinary Parasitology*. **100**, 13–20.

Vincourt, J.-B., Gillet, P., Rat, A.-C., Guillemin, F., Netter, P., Mainard, D., & Magdalou, J. (2012). Measurement of matrilin-3 levels in human serum and synovial fluid using a competitive enzyme-linked immunosorbent assay. *Osteoarthritis and Cartilage*. **20**, 783–786.

Volanakis, J. (2001). Human C-reactive protein: expression, structure, and function. *Molecular Immunology,*. **38,** 189–197.

Volanakis, J.E., Kaplan, M. H. (1971). Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proceedings from the Society for Experimental*

Biology and Medicine. **136**, 612–614.

Vowinkel, T., Mori, M., Krieglstein, C. F., Russell, J., Saijo, F., Bharwani, S., Turnage, R. H., Davidson, W. S., Tso, P., Granger, D. N., & Kalogeris, T. J. (2004). Apolipoprotein A-IV inhibits experimental colitis. *Journal of Clinical Investigation*. **114**, 260–269.

Wang, H. T., Li, Y. H., Chou, I. P., Hsieh, Y. H., Chen, B. J., & Chen, C. Y. (2013). Albusin B modulates lipid metabolism and increases antioxidant defense in broiler chickens by a proteomic approach. *Journal of the Science of Food and Agriculture*. **93**, 284–92.

Wang, Y., George, S. P., Srinivasan, K., Patnaik, S., & Khurana, S. (2012). Actin reorganization as the molecular basis for the regulation of apoptosis in gastrointestinal epithelial cells. *Cell Death and Differentiation*. **19**, 1514–24.

Wang, Y., Guo, Y., Ning, D., Peng, Y., Cai, H., Tan, J., Yang, Y., & Liu, D. (2012). Changes of hepatic biochemical parameters and proteomics in broilers with cold-induced ascites. *Journal of Animal Science and Biotechnology*. **3**, 41-50

Wang, Y., Srinivasan, K., Siddiqui, M. R., George, S. P., Tomar, A., & Khurana, S. (2008). A novel role for villin in intestinal epithelial cell survival and homeostasis. *The Journal of Biological Chemistry*. **283**, 9454–64.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L., & Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *Electrophoresis*. **16**, 1090–4.

Weeks, C. A., Danbury, T. D., Davies, H. C., Hunt, P., & Kestin, S. C. (2000). The behaviour of broiler chickens and its modification by lameness. *Applied Animal Behaviour Science*. **67**, 111–125.

Weinberg, R. B. (2002). Apolipoprotein A-IV polymorphisms and diet - gene interactions. *Genetics and Molecular Biology*. **81**, 125–134.

Weinberg, R. B., Anderson, R. a, Cook, V. R., Emmanuel, F., Denefle, P., Hermann, M., & Steinmetz, A. (2000). Structure and interfacial properties of chicken apolipoprotein A-IV. *Journal of Lipid Research*. **41**, 1410–8.

Westermeier, R., Naven, T., & Hopker, H. R. (2008). *Proteomics in Practice*. Weinheim, Germany: Wiley-VCH.

Wicher, K. B., & Fries, E. (2006). Haptoglobin, a hemoglobin-binding plasma protein, is present in bony fish and mammals but not in frog and chicken. *Proceedings of the National Academy of Sciences of the United States of America*. **103**, 4168–73

Wicher, K. B., & Fries, E. (2010). Evolutionary aspects of hemoglobin scavengers. *Antioxidants & Redox Signaling*. **12**, 249–59.

Williams, J. (1968). A comparison of glycopeptides from the ovotransferrin and serum transferrin of the hen. *The Biochemical Journal*. **108**, 57–67.

Wilson, R. (2013). Sensitivity and specificity: twin goals of proteomics assays. Can they be combined? *Expert Review of Proteomics*. **10**, 135–149.

Worboys, J. D., Sinclair, J., Yuan, Y., & Jørgensen, C. (2014). Systematic evaluation of quantotypic peptides for targeted analysis of the human kinome. *Nature Methods*. **11**, 1041–4.

Xie, A. H., Newberry, L., Clark, F. D., Huff, W. E., Huff, G. R., Balog, J. M., … Balog, A. J. M. (2002). BChanges in Serum Ovotransferrin Levels in Chickens with Experimentally Induced Inflammation and Diseases Changes in Serum Ovotransferrin Levels in Chickens with Experimentally Induced Inflammation and Diseases. *Avian Diseases*. **46**, 122–131.

Xie, H., Huff, G. R., Huff, W. E., Balog, J. M., Holt, P., & Rath, N. C. (2002). Identification of ovotransferrin as an acute phase protein in chickens. *Poultry Science*. **81**, 112–20.

Xie, H., Huff, G. R., Huff, W. E., Balog, J. M., & Rath, N. C. (2002). Effects of ovotransferrin on chicken macrophages and heterophil-granulocytes. *Developmental & Comparative Immunology*. **26**, 805–815.

Xie, H., Rath, N. C., Huff, G. R., Huff, W. E., & Balog, J. M. (2000). Effects of Salmonella typhimurium lipopolysaccharide on broiler chickens. *Poultry Science*, **79**, 33–40.

Yozo, G., Salih, E., Glimcher, M.J., & Gerstenfeld, L. C. (2002). Characterization of the Major

Non-collagenous Proteins of Chicken Bone: Identification of a Novel 60-kDa Non-collagenous Phosphoprotein. *Biochemical and Biophysical Research Communications*. **208**, 863–870.

Yamauchi, K., & Ishihara, A. (2009). Evolutionary changes to transthyretin: developmentally regulated and tissue-specific gene expression. *The FEBS Journal*. **276**, 5357–66.

Yan, N. (2013). Structural advances for the major facilitator superfamily (MFS) transporters. *Trends in Biochemical Sciences*. **38**, 151–159.

Yan, S., & Sloane, B. F. (2003). Molecular regulation of human cathepsin B: implication in pathologies. *Biological Chemistry*. **384**, 845–54.

Yazdani, A., Asasi, K., & Nazifi, S. (2015). Evaluation of acute-phase proteins and inflammatory mediators changes in native chickens experimentally infected with Salmonella typhimurium. *Comparative Clinical Pathology*. **24**, 733–739.

Zekarias, B., Ter Huurne, A. A. H. M., Landman, W. J. M., Rebal, J. M. A., Pol, J. A. P., & Gruys, E (2002). Review article Immunological basis of differences in disease resistance in the chicken. *Veterinary Research*. **33**, 109–125.

Zhang, J., Li, C., Tang, X., Lu, Q., Sa, R., & Zhang, H. (2015). High concentrations of atmospheric ammonia induce alterations in the hepatic proteome of broilers (Gallus gallus): An iTRAQ-based quantitative proteomic analysis. *PLoS ONE*. **10**, 1–18.

Zhang, J., Li, C., Tang, X., Lu, Q., Sa, R., & Zhang, H. (2015). Proteome changes in the small intestinal mucosa of broilers (Gallus gallus) induced by high concentrations of atmospheric ammonia. *Proteome Science*. **13**, 9.

Zhang, L., Ma, L., Liu, R., Zhang, Y., Zhang, S., Hu, C., Song, M., Cai, J., & Wang, M. (2012). Eimeria tenella heat shock protein 70 enhances protection of recombinant microneme protein MIC2 subunit antigen vaccination against E. tenella challenge. *Veterinary Parasitology*. **188**, 239–46.

Zheng, A., Luo, J., Meng, K., Li, J., Zhang, S., Li, K., Cai, H., Liu, G., & Yao, B. (2014). Proteome changes underpin improved meat quality and yield of chickens (Gallus gallus) fed the probiotic Enterococcus faecium. *BMC Genomics*,. **15**, 1–14.

Zhi, W., Wang, M., She, J. X. (2011). Selected reaction monitoring (SRM) mass spectrometry without isotope labeling can be used for rapid protein quantification. *Rapid Communications in Mass Spectrometry*. **25**, 1583–1588.

Zou, W., Ke, J., Zhang, A., Zhou, M., Liao, Y., Zhu, J., Zhou, H., Tu, J., Chen, H., & Jin, M. (2010). Proteomics analysis of differential expression of chicken brain tissue proteins in response to the neurovirulent h5n1 avian influenza virus infection. *Journal of Proteome Research*. **9**, 3789–3798.