Acute phase proteins as a biomarker of health and disease in chickens

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Author's Declaration

This thesis and work contained within it, was conducted from October 2014 to September 2018 under the supervision of Professor David Eckersall and Professor Maureen Bain, of the University of Glasgow. The work presented in this thesis was performed solely by the author except where the assistance of others is acknowledged below; a) Dr Richard Burchmore Glasgow Polyomics carried out and provided the proteomic analysis of serum samples described in chapter 4.6. b) Ms Emily Klmj from Temasek Polytechnic assisted with the real time PCR described in chapter 5. c) Dr Ruedi Nager assisted with the statistical modelling and analysis described in chapter 6.

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Abstract

Animals undergoing a challenge to their state of health mount a vigorous response which involves both the innate and acquired immune systems. The varied non-specific responses of an animal to infection, inflammation or trauma are collectively referred to as the acute phase response (APR). The APR is a very complex reaction involving both local and systemic effects. Acute-phase proteins (APP) are a group of blood proteins primarily synthesised in the liver in response to pro-inflammatory cytokines being released primarily from leukocyte activation during an APR. The use of APP for diagnostic purposes in both human and veterinary medicine has increased greatly within the last decade.

The aim of this thesis was to investigate the APR in chickens in response to i) vaccination using a Newcastle disease and Infectious Bronchitis (N/B) Live, freeze-dried virus vaccine; ii) an experimental challenge with Poultry red Mite (PRM); (iii) an LPS challenge experiment with an *E. coli* LPS. Four APP namely serum amyloid A (SAA), alpha 1 acid glycoproteins (AGP), ovotransferrin (OVT) and ceruloplasmin (CP) were studied in detail along with other blood component including heterophile / lymphocyte (H/L) ratios and corticosterone. For SAA, AGP and CP, species specific ELISA kits are now commercially available, but these had not previously been validated. The results from chapter 2 demonstrated that all three ELISAs gave good accuracy, had a suitably low detection limit and allowed discrimination between different levels of APP in chicken samples. For OVT a lab based radial immunodiffusion assay (RID) was used.

The results of the current study have shown that a mild response of APP (SAA and AGP) in SPF chick post vaccination at day one and two post vaccination. H/L ratios also increased, and this measure was deemed more sensitive and consistent in terms of measuring the mild stress response under the conditions employed than the APP though of these the SAA was the most promising.

In the other experimental challenge with PRM in laying hens, the serum levels of SAA were also significantly increased, and this was subsequently found to be positively correlated with the level of PRM infestation which was monitored for several months, further serum protein profile altarations were decsibred in this study. Whereas, LPS challenge induced significant with high magnitude of APP (SAA, AGP and OVT). In addition, local expression of APP have been investigated and significant changes of other blood components were disecribed. Looking for the APR by non invasive way in chickens challenged with LPS, there were interesting sensitive detection of the body surface temperature. Also, a significant correlation of the cloacal with the body surface temperature changes in develpment of pyrexia.

In summary, the research presented in this thesis has demonstrated that SAA is the most sensitive APP and therefore potentially the most useful biomarker in chickens (layers and broilers): this APP increased more rapidly and by a greater magnitude than the other APP following a range of stimuli. AGP and OVT moderately increased and usually peaked later than SAA, while CP rarely changed.

Further research is needed to investigate the relationship between the local and systemic APR and to determine the significance of extrahepatic versus hepatic production of APP in the chicken and, the associated H/L ratio and thermal imaging in early disease recognition as both of these measurements proved to be useful in such studies.

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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:

APP in response to vaccine N/B (Chapter 3)

Haider Kaab, Maureen M. Bain, and Peter David Eckersall (2017). Acute phase proteins and stress markers in the immediate response to a combined vaccination against Newcastle disease and infectious bronchitis viruses in specific pathogen free (SPF) layer chicks. 2017 Poultry Science 0:1-7 http://dx.doi.org/10.3382/ps/pex340

LPS challenge (Chapter 5)

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PRM and response of APP (Chapter 4)

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Conference proceedings

2015 69th Annual Conference of the Association for Veterinary Teachers and Research Work, (AVTRW)
Acute phase proteins in Chickens: Validation of Enzyme Linked Immunosorbent Assays for 1-acid Glycoprotein, and Serum Amyloid A, Royal veterinary college, London, UK

2016 XXV Annual Conference of World's Poultry Science Association, (WPSA)
Effects of administering a routine vaccination on the acute phase protein response in SPF layer chicks, Beijing China.

2017 World Poultry Science Association (WPSA) UK Annual Spring Meeting Egg-Meat.
Protein fractional synthetic rate of different tissues in broiler chicks determined by an oral tracer procedure. Edinburgh, UK.

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
1D	One-dimensional
2D	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
C	Control
Ca^{2+}	Calcium ion
CHAPS	3[(cholamidopropyl)dimethylammonio]-1-propane sulphonate
Cl-	Chloride ion
CRP	C-reactive protein
CP	Ceruloplasmin
Cu	Copper
dH20	Distilled water
ELISA	Enzyme linked immunosorbent assay
MS	Mass spectrometry
g	Gram
g/L	Gram per litter
HDL	High density lipoprotein
h	hour
Hp	Haptoglobin
Hp	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
kDa	Kilo Dalton
L	Litre
LC	Liquid chromatography
LCMS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LPAI	Low pathogenic avian influenza
LPS	Lipopolysaccharride
M	Molar
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
MS/MS	mass spectrometry
Mw	Molecular weight
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
n	Sample size
Na2CO3	Sodium carbonate
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NaOH	Sodium hydroxide
NCP	Nitroceullulose paper
No.	Number
Ovt	Ovotransferrin
PAGE	Polyacrylamide gel electrophoresis
pH	Power of hydrogen
PO42-	Phosphate ion
p-value	Probability that null hypothesis is true
QC	Quality control
RID	Radial immunodiffusion
S	Saline
SAA	Serum amyloid A
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

TBS-T	Tris-buffered saline with Tween 20
TIBC	Total iron binding capacity
TNF	Tumour necrosis factor
TBS	Tris buffered saline
V	Volt
vs.	Versus
wks	weeks

Chapter 1 General Introduction

1.1 The Poultry Industry

In 2017, the world's broiler meat production amounted to about 90.7 million metric tons, and is forecasted to increase to about 92.47 million metric tons by the end of following year : (https://www.statista.com/statistics/237637/ Production-of- poultry-meat-worldwide-since-1990). In the UK alone, there were over 916 million broiler chickens, 162 million turkeys and 100,000 geese farmed for meat in the UK. According to the UK Department for Environment, Food and Rural Affairs (DEFRA), this equates to a 2% increase in broiler production from the previous year, with total broiler chicken production reaching 1.42 million tonnes of meat in 2017.

Poultry meat is considered healthier as it is less fat content compared to other meat types (Marangoni et al., 2015). Therefore, the increased demand for broiler meat on a worldwide basis has only been met because of the impressive progress that has been made by primary breeding companies in the genetic selection (Buzala et al., 2015) for improved growth rate, skeletal health (Julian, 2005), food conversion ratio (Nangsuay et al., 2015) and disease resistance (Parmentier et al., 2010), together with improvements in management. Thus, the length of the grow out period has been halved from 10.5 wks to only 5 wks with typical slaughter weights of 2.2kg and a food conversion ratio of 1.5 compared to those reported in 1953 (Siegel, 2014). This scaling up of the industry has significantly reduced production costs, resulting in cheaper meat for the consumer.

Another important sector in poultry industry is the egg production. Egg constitute one of the most affordable source of animal protein available, and so it is no surprising that the number of the laying flocks are rapidly increasing in develop countries such as India and China (Bain et al., 2016). Over past decade from 2000 -2014, world egg production has increased by 36.5 %, or an average of 2.8 % per year (https://www.wattagnet.com/poultry-market-data). In 2014, a lying hens flock of 7.2 billion hens produced around 70 million metric tons. In the UK, egg production quarterly figures for 2017 show 7.5 million packs of eggs (DEFRA). This increase in egg production is due to improvements in several sectors such as genetic and nutrition, the use of artificial lighting

programmes and rearing system has seen the number of the eggs per hen rise from 130 per annum in 1940 to more than 300 per annum by 2000 (Rossi et al., 2013).

1.2 Challenges facing the poultry industry

As poultry production continues to grow, infectious diseases remain a threat to the industry (Goryo et al., 1987; Normile, 2004; Welchman et al., 2010; Belayheh et al., 2015). 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 growth rates and negatively affecting productivity. As well as impacting negatively on production, disease is also detrimental to bird welfare. The levels of economic losses to the poultry industry from poultry diseases will be in the order of 10% of industry (Biggs, 1982; Brown Jordan et al.,2018). This figure includes the costs of vaccinations and medications as well as the costs of mortalities from diseases, primarily those from infectious causes. The economic losses could be higher up to 20%, as the effects of subclinical disease which causes suboptimal production during the life of these poultry flocks (Bagust, 2012).

Disease problems being caused by single pathogens are seen far less now in developed intensive poultry industries, a partial exception being some free-range poultry. However mixed infections, complex interactions with the production environment and poor production from disease complexes are common particularly respiratory diseases, enteric disorders and immunosuppressive infections which will predispose to infection by other pathogens whether viral, mycoplasmas or bacterial. (Bagust, 2012). 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 (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.

1.3 The Acute Phase Response

The production of chicken meat and eggs has increased dramatically in recent years, but this has not come without consequences especially in relation to disease control. The host innate immune response (IIR) precedes and initiates the acquired immune response to disease challenge (Janeway, 2001). It is diverse and includes physical barriers, activation of phagocytic cells, the complement system, and receptors that serve to prevent infection, eliminate potential pathogens and initiate the inflammatory process (Janeway, 2001). The host innate 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 specific to a particular pathogen in the way that the adaptive immune responses are (Kogut, 2009). The relatively recent discovery that the IIR directs the acquired response supports efforts to select poultry with an efficient early IIR (Zekarias et al., 2002). The acute phase response (APR) is an important part of the IIR and is summarised in (Figure 1.1). The APR is complex and involves both systemic and local effects. An APR will be initiated as a result of infection, inflammation, trauma or tissue injury or by the introduction of immunogens such as a bacterial lipopolysaccharide (LPS), turpentine or vaccination (O'Reilly and Eckersall, 2014). Induction of the APR is dependent on the formation of pro-inflammatory signals (cytokines and chemokines), which are generated by activation of macrophages, monocytes, fibroblasts, platelets, keratinocytes, endothelial cells and T-cells (Janeway, 2001). In mammals there are three major pro-inflammatory cytokines; interleukins-1 and -6 (IL-1 and IL-6), and tumour necrosis factor (TNF-α); these are largely responsible for the systemic effects of the APR (Cray et al., 2009). Cytokines are proteins of low molecular weight that are released in response to activation of macrophages, lymphocytes, Kupffer cells and fibroblasts. The systemic effects of cytokines include fever (Dinarello, 1983; Nielsen, et al., 1998), anorexia (Klasing and Peng, 1987; Nielsen et al., 1998), glucocorticoid release and activation of both the complement and the clotting cascade. The release of cytokines also leads to decreased serum zinc, iron and calcium (Luthman et al., 1991) and induces the synthesis and release of APP from the liver (Cray et al.,2009)

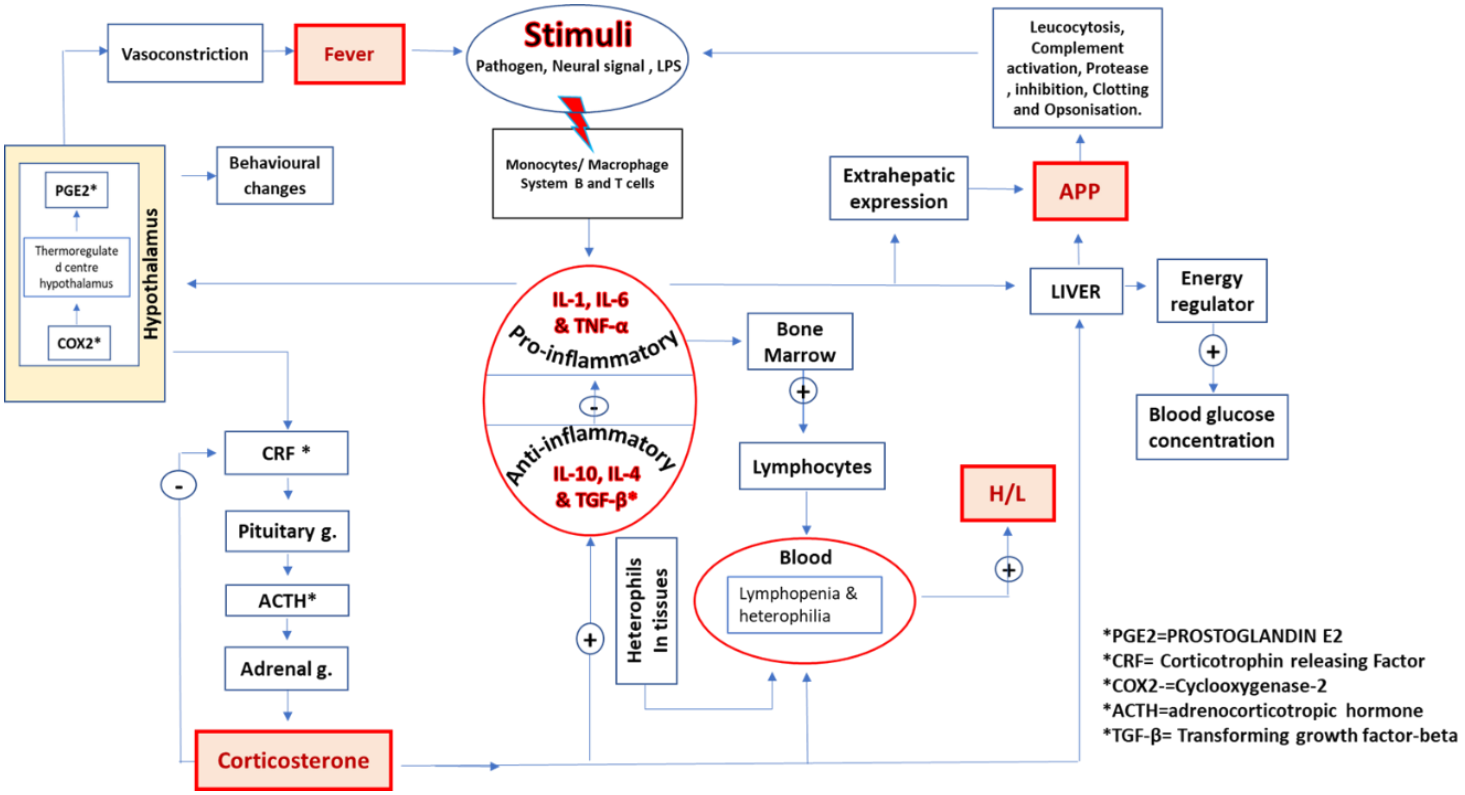

Figure 1.1 Acute phase response (chickens) and associated effects and outcomes
A hypothetical stimulus triggers various target cells at the site of infection or injury to produce cytokines (pro- and anti-inflammatory). Pro-inflammatory cytokines (not all cytokines are shown here) induce APP to be released from hepatocytes and in some instances, there is also a local tissue response. Pro-inflammatory cytokines also induce the release of ProstaglandinE2 from the hypothalamus, which results in pyrexia. Conversely, the hypothalamus via the pituitary induces the adrenal glands to release corticosterone. Corticosterone plays an immuno-modulator role by up regulating the responses of anti-inflammatory agents and downregulating the pro-inflammatory cytokines. Part of this response is the influence on blood components leading to lymphopenia and heterophilia which consequently increases the Heterophil/Lymphocyte (H/L) ratio. Corticosterone release also induces the release of glucose from the liver so limiting the effects of anorexia. (Cray et al., 2009, 2012; Kogut et al., 2002; Klasing and Johnstone, 1991; Walter, et al., 201

The inflammatory cytokine, IL-6, stimulates the synthesis of most of the APP (Chamanza et al., 1999b), via its direct influence on hepatocytes (Sehgal et al., 1989). Cytokines can also cause the local release of APP from a wide range of extrahepatic tissues which enhances the APR and so are integral in the whole inflammatory process (Schrodl et al., 2016). The extrahepatic release of APP has not been widely studied in mammals or birds (Berg et al., 2011; Lecchi et al., 2012; Marques et al., 2017; Vernooy et al., 2005) but it is thought that this source of APP plays an important role in local inflammatory reactions (Inforzato et al., 2006; Schrodl et al., 2016). In birds just one recent study has been done which conducted in normal and healthy broilers chickens (Marques et al., 2017). This study has suggested that many tissues other the liver express APP, however the expression of APP were in low concentration.

Production of cytokines IL-1β, IL-6, IL-17 family and TNF-α have been documented in poultry (Franchini et al., 2004; Giansanti et al., 2006). Injection of partially purified forms of these cytokines has been shown to induce multiple APP effects in addition to causing pyrexia and anorexia in chickens. IL-6 for example has been shown to initiate the liver to produce APP (Juul-Madsen et al., 2007; Klasing and Johnstone, 1991), and TNF-α has been shown to increase tumour cytolysis activity and to initiate cartilage resorption (Juul-Madsen et al., 2007; Klasing and Peng, 1987).

1.4 Production of acute phase proteins

During the APR dramatic changes in serum concentration occur due to rapid production of so-called “positive” APP and deceased production of “negative” APP by hepatocytes. The positive APP are further classified into major, moderate and minor categories (Eckersall, 2008). Positive APP are important in optimisation and trapping of microorganisms, in activating the complement system, in binding cellular remnants, in neutralising enzymes savaging free haemoglobin and radicals and modulating the host immune response. Major positive APP increase 10-100-fold, often within the first 48h post the triggering event. This is often followed by a rapid decline because of their short half-life (Ceron et al., 2005). Moderate positive APP increase in concentration between 4-10-fold, while minor APP increase less than 2-3-fold (Paltrinieri, 2008). Moderate and minor positive APP tend to follow the magnitude of the response and their increase may occur more slowly and be of longer

duration (chronic inflammation) depending on the severity of triggers (Ceron et al., 2005). The APP pattern varies from one species to another. For example, C-Reactive protein is a major APP in human and dog but its concentration does not modify the APP in cattle or cats (Cray, 2012) and it is uncharacterised in chickens (O'Reilly, 2016).

In chickens, serum amyloid A (SAA) (Alasonyalilar et al., 2006; Nazifi et al., 2011) is the only major APP in chickens, α1-acid glycoprotein (AGP) and Ovotransferrin behave as moderate APP, Ceruoplasmin (CP) and PIT54 are regarded as minor APP (O'Reilly, 2016). The biological functions of APP have been reviewed in mammals by many researchers (Ceron et al., 2005; Murata et al., 2004; Petersen et al., 2004), and in poultry by O'Reilly and Eckersall (O'Reilly and Eckersall, 2014) will be descused in section 1.8.

1.5 Glucocorticoids and corticosterone

Another aspect of the APR is production of glucocorticoids (Figure 1.1). The role of corticosterone in poultry is the same as cortisone in mammals i.e. including stimulating and modulating proteins synthesized in the liver as part of the APR (Amrani et al., 1986). Glucocorticoids increase during the APR as a result of cytokine-stimulation of the pituitary-adrenal axis, which increases secretion of adrenocorticotrophic hormone (Klasing and Johnstone, 1991). Thus, an increase of corticosterone is noticed later than the appearance of IL-6 (Amrani et al., 1986). It has been reported that administration of corticosterone to the fowl can induce APP synthesis even in the absence of an inflammatory response (Curtis and Butler, 1980), which suggests that glucocorticoids may work independently of cytokines. In other words, corticosterone has an additive effect to the cytokine stimulation of APP synthesis following induced inflammation (Chamanza et al., 1999b). Glucocorticoids are also considered to be an important factor mediating the high concentration of APP in non-inflammatory situations such as handling stress of birds (Chamanza et al., 1999b).

1.6 Heterophil/Lymphocyte (H/L) Ratio

As part of the inflammatory response, neutrophils or heterophils (in birds) are activated by cytokines to increase their phagocytic and bactericidal activity

(Andreasen et al., 1991; Kogut et al., 2002) (Figure 1.1). Elevation of blood corticosterone following the administration of exogenous compounds or as a consequence of stress (physiologic, management, or pathological causes) leads to an increase in the H/L ratio (Maxwell, 1993; Davis and Maerz, 2008). It has been pointed out that the corticosterone response and H/L ratio vary in sensitivity according to different stressors. For example, the H/L ratio is a sensitive indicator in cases of mild to moderate stress, but it is not a good indicator of severe stress as in the latter situation there is a decrease in heterophils (H) and an increase in lymphocytes (L) with a consequently low H/L ratio (Maxwell, 1993).

Inconsistency in responses of corticosterone and the H/L ratio to various stressors may be related to some factors such as repeated stress events, when corticosterone returns to a low concentration (Rich and Romero 2005). This may not be the case for the H/L ratio (McFarlane and Curtis, 1989). Plasma corticosterone increases instantaneously after the onset of stress (Jones et al., 2016), whereas the H/L ratio is an indicator of stress over a longer time period (Shini et al., 2008).

1.7 Prostaglandin E2 and Pyrexia

Pyrexia or fever (Axelrod and Diringer, 2008) is a common clinical sign of disease characterized by an elevation of temperature above the normal range due to an increase in the body temperature regulatory set point (Karakitsos and Karabinis, 2008). Pyrexia is a prominent component of many inflammatory and immunologically mediated diseases. Pro-inflammatory cytokines in the systemic circulation causes a release of prostaglandin E2 (PGE2) in the hypothalamus. PGE2 is the ultimate mediator of the febrile response (Netea et al., 2000) (Figure 1.1). The set-point temperature of the body will remain elevated until PGE2 is no longer present which acts on the hypothalamus. Consequently generates a systemic response back to the rest of the body, causing heat- creating effects to match a new temperature level. In many respects, the hypothalamus works like a thermostat (Dinarello, 2004; Watanabe et al., 1997).

1.8 Acute phase proteins in chickens

There are many types of APP in chickens (O'Reilly and Eckersall, 2014), and in the context of this thesis the focus will be on Serum amyloid A (SAA), Alpha acid

glycoprotein (AGP), Ovotransferrin (OVT) and Ceruloplasmin (CP). These have been chosen firstly because they are regarded as the most important APP in chickens (Table 1.1 and O'Reilly, 2016) Secondly, because species specific commercial ELISA assays for SAA, AGP and CP have recently become available, albeit they have not been validated, and for OVT a laborotory based radial immunodiffusion assay has recently been developed (O'Reily, 2016). These assays make investigation of these four APP more feasible as discussed later (section 1.8.1).

1.8.1 Serum amyloid A (SAA)

SAA is a major APP in chickens and is also found in most other species where levels have been shown to dramatically increase in response to inflammation by more than 1000-fold, in order to play an essential role in the defense mechanism (Uhlar and Whitehead, 1999). The main function of SAA is to modify the transport and metabolism of lipoprotein in APR to allow cholesterol to access damaged tissues for repair and reconstruction of damaged membranes and to clear lipid debris formed by bacteria and damaged tissues (Landman and Gruys, 1998). SAA also has a modulatory role, inhibiting pyrexia and pro-inflammatory events during the APR (Shainkinkestenbaum et al., 1991; Uhlar and Whitehead, 1999). Chickens with infectious bursal disease (IBD) showed significant increases in SAA, which was found to be the most sensitive parameter among the APP: Ceruloplasmin, fibrinogen, and Haptoglobin/PIT54 (Nazifi et al., 2010). These authors found a strong relationship between the severity of the disease and the effect of APP changes. The basal level of SAA in laying hens has been measured by non-species-specific ELISA and was found to be 0.154 ± 0.02 µg/ml (Alasonyalilar et al., 2006). Others reported basal levels of 1.590 ± 0.0041 µg/ml (Nazifi et al., 2010). SAA has been shown to be significantly higher in birds subjected to experimental infection with infectious bronchitis virus compared to a non-infected control group, and there was a significant negative correlation between Haptoglobin /PIT54 and SAA in the non-infected group (Nazifi et al., 2011).

1.8.2 Alpha 1-acid glycoprotein (AGP)

The main function of AGP is to act as a natural anti-inflammatory factor and neutralize the toxicity of bacterial LPS by binding to protein (Murata et al., 2004). Measuring AGP has been used successfully to monitor the inflammatory process in

cattle (Carter et al., 2002). It can be measured by radial immunodiffusion assay (Tamura et al., 1989), with base level of AGP in chickens blood being between 0.2 - 0.3 mg/ml using this method (Koppenol et al., 2015; Takahashi et al., 1998), while the basal level of AGP measured by ELISA has been reported to be between 0.52 – 0.58 mg/ml (Peebles et al., 2014).

Elevated serum AGP levels have been reported in pigs with infectious diseases such as pneumonia or meningitis (Itoh et al., 1993). AGP has been described as a moderately positive APP in cases of hepatitis or in response to experimental turpentine administration in dogs (Sevelius and Andersson, 1995; Yamashita et al., 1994). In cats, measuring AGP is clinically important in the differential diagnosis of feline infectious peritonitis (Duthie et al., 1997). In chickens AGP is classified as a moderate APP (O'Reilly and Eckersall, 2014), and it is widely measured in research areas such as nutrition (Takahashi et al., 2009; Takahashi et al., 1995). It has been well documented that serum levels of AGP increase in response to bacterial or viral infections, thus, monitoring this APP can be used to detect infection, particularly in subclinical cases which may subsequently increase in severity in stressed animals (Holt and Gast, 2002). It has been demonstrated that the level of AGP significantly increased in *Escherichia coli (E. coli)* infections (Nakamura et al., 1998) or after injection within *Salmonella Typhimurium* (Adler et al., 2001). In viral infection, the level of AGP has been shown to be increased significantly in both infectious bronchitis and infectious laryngotracheitis (ILT), but the level of AGP increased higher in the former compared with the latter (Nakamura et al., 1998). The levels of AGP increased with viral virulence in the cases of bursal disease virus (Inoue et al., 1997).

1.8.3 Ovotransferrin (OVT)

In chickens, this APP is produced in the oviduct under the influence of oestrogen for incorporation into the egg and in the hepatocytes for secretion into blood in response to pro-inflammatory cytokines (Hallquist and Klasing, 1994). The main function of OVT is storage, binding and transferring of iron (Aguilera et al., 2003). Although OVT is a moderate positive APP in chickens, the increment of OVT at 72 h post induced inflammation has been recorded around 5 times higher than that of the control group (Xie et al., 2002a). In mammals, OVT has a different response, during APR, compare to chickens, where it is considered a negative APP in mammals (Ceron et al., 2005).

OVT is a major glycosylated protein in chickens, and it has bacteriostatic functions that act to deprive bacteria from the iron which is important to their growth (Xie et al., 2002a).

OVT was elevated in chickens in response to inflammation by infectious pathogens or tissue injury (Rath et al., 2009), but not in non-inflammatory conditions, such as skeletal metabolic disorder and femoral head disorder (Durairaj et al., 2009). In a study conducted to measure the difference in level of APP between vaccinated and unvaccinated chickens after challenge with a low pathogenic avian influenza virus (LPAI), it was shown that the plasma levels of OVT were significantly less in the vaccinated compared to the unvaccinated chickens at 48 and 96 hours post challenge (Sylte and Suarez, 2012). OVT appears to remain elevated as long as the inflammation continues (Rath et al., 2009; Xie et al., 2002b), and it may act as an immunomodulator to prevent microbial growth (Xie et al., 2002a) and as an antioxidant agent (Superti et al., 2007). OVT may therefore have a protective role and be responsible for restoring homeostasis in chickens (Cermelli et al., 2000). Thus, OVT can be used as a diagnostic biomarker of infection and inflammation in chickens (Xie et al., 2002).

1.8.4 Ceruloplasmin (CP)

The first description of CP as an APP in chickens was reported by Butler and his co-workers in 1972 (Butler et al., 1972), following post challenge with *E. coli* LPS which induced an increased CP levels of up to five-fold at 24 h post-injection. These authors measured CP by para-phenylenediamine oxidase activity (Butler et al., 1972). The basal level of CP was estimated it was 23.1 mg/L (Nazifi et al., 2010). The main function of CP is to transfer and store copper within the body, and it also has anti-oxidative functions (Floris et al., 2000). CP has been reported to increase significantly after *Eimeria* infection in poultry (Richards and Augustine, 1988), while other researchers found that the CP levels significantly decreased after Gumboro infection (Nazifi et al., 2010).

1.8.5 Other relevant APP in chickens

Chickens represent a vital 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.

Other types of APP than these mentioned above, have been investigated in chickens such as albumin which 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). Albumin plays as a regulator of plasma osmotic pressure, can bind /transport endo/exogenous compounds and has antioxidant properties (Sitar et al. 2013).

Mannan binding lectin (MBL) is APP and a 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). which binds microorganisms, phagocytosis, plays an immunomodulatory and has ability to activate complement system through distinct MBL pathway (Nielsen et al., 1999; Juul-Madsen et al., 2003; Schou et al.,2010).

Other kind of APP include, α-1 microglobulin named PIT54 in avian species (Wicher and Fries, 2006). PIT54 is one of the plasma proteins bind free Hb or its haem group, blocking its detrimental effects and mediating its removal from the blood stream (Wicher and Fries, 2010).

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). Plasma FN is a minor APP, increasing during an APR and able to diffuse into tissues and form part of the fibrillar matrix, where together with tissue FN, it plays an important and active role in wound healing (Labat-Robert, 2012). Fibronectin 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). In chickens, found plasma FN levels increased as a result of turpentine

injection (Amrani et al. 1986), also consequent to elevated temperature (42°C) for three hours (JianHua et al., 2000). In response to ACTH and dexamethasone administration, FN in chicken plasma increased (LiCheng et al., 2000).

Table 1.1 APP response to different pathogenic stimuli
Studies of bacterial viral and parasitic diseases where APP have been measured (Modified and adapted from O'Reilly and Eckersall, 2014).

Bacterial agents	APP	Reference
Salmonella ser Typhimurium (LPS)	Ceruloplasmin CP	(Koh et al., 1996)
	Ovotransferrin OVT	(Xie et al., 2000)
	Haemopexin HX	(Adler et al., 2001)
	Haptoglobin PIT54	(Millet et al., 2007)
	Mannan binding lectin MBL	(Millet et al., 2007)
	SAA	(Yazdani et al., 2015)
Salmonella ser Gallinarum	Ceruloplasmin CP	(Garcia et al., 2009)
	Haptoglobin PIT54	(Garcia et al., 2009)
	Ovotransferrin OVT	(Garcia et al., 2009)
	Haemopexin HX	(Garcia et al., 2009)
Escherichia coli (LPS)	Ceruloplasmin CP	(Butler et al., 1972)
	Ovotransferrin OVT	(Takahashi et al., 2009)
		Rath, et al., 2009
	Acid- Glycoprotein AGP	(Xie et al., 2002a)
		(Hallquist and Klasing, 1994)
		(Nakamura et al., 1998)
	Haemopexin HX	(Buyse et al., 2007)
	C-reactive protein CRP	(Barnes, 2001)
		(Buyse et al., 2007)
		(Patterson.Lt and Mora, 1965)
Viral agents	APP	Reference
Infectious bronchitis virus	Serum amyloid –A SAA	(Nazifi et al., 2011)
	Ovotransferrin OVT	(Xie et al., 2002a)
	Acid- glycoprotein AGP	(Nakamura et al., 1996)
	Mannan binding lectin MBL	(Juul-Madsen et al., 2003)
	SAA and hepatoglobulin Hp	

		Asasi et al.2013
	SAA and AGP	Kaab et al., 2018
Infectious bursal disease virus **Gumboro disease**	Ceruloplasmin CP	(Nazifi et al., 2010)
	Serum amyloid –A SAA	(Nazifi et al., 2010)
	Haptoglobin PIT54	(Nazifi et al., 2010)
	Fibrinogen FB	(Nazifi et al., 2010)
	Ovotransferrin OVT	(Xie et al., 2002b)
	Mannan binding lectin MBL	(Nielsen et al., 1999)
Low pathogenic avian influenza	Acid- glycoprotein AGP	(Sylte and Suarez, 2012)
Infectious laryngotracheitis virus	Ovotransferrin OVT	(Xie et al., 2002a)
Fowl Poxvirus	Ovotransferrin OVT	(Xie et al., 2002a)
Respiratory enteric orphan virus	Ovotransferrin OVT	(Xie et al., 2002a)
Parasites agents	APP	Reference
Dermanyssus gallinae (Red mite) in laying hens	SAA, AGP, OVT and OVT	Kaab *et al.*, 2018 (In press)
Eimeria maxima ; Eimeria tenella	Ovotransferrin OVT	(Rath et al., 2009)
Eimeria acervulina	Ceruloplasmin CP	(Richards and Augustine, 1988)

1.9 Measuring APP

Automated biochemical analysers are commonly used machines to measure total blood protein and albumin for basic health assessment (Cray et al., 2009). For more than 40 years, protein electrophoresis has been used to study APP in both human and veterinary medicine with many APP being within the α- and ß-globulins of serum (Sandor, 1966; Alper, 1974; Eckersall, 2008). Protein electrophoresis provides a precise measure of the albumin: globulin ratio and can therefore be used as a useful if rather imprecise means of monitoring the acute and chronic progression of the APP (Gentry and Lumsden, 1978; Stokol et al., 2001). The disadvantage of this technique is that changes in proteins with low concentrations may not be noticed

(Cray et al., 2009). ELISAs, radioimmunoassay, Western blot, and mRNA analyses can be used to measure individual APP (Ceron et al., 2005; Eckersall, 1995; Paltrinieri, 2008; Schreiber et al., 1989).

These immunoassays form the backbone of tests used in the study of infectious diseases and clinical endocrinology, and hence can be used to identify causative agents of disease, assess the extent and nature (epidemiology) of disease, and measure hormones at physiological levels (Aydin, 2015). These assays are versatile, having been applied to a wide range of biological fields, for example, viruses, bacteria, fungi, and protozoan and metazoan parasites (Aydin, 2015). Since 1971 (Engvall and Perlmann, 1971), when the first effective enzyme-labelled assay was described, thousands of applications have been published dealing with the quantification of antigens and antibodies for research and applied purposes (Aydin, 2015). ELISA and related assays involving use of enzymes to obtain colorimetric results have now replaced radioimmunoassay (RIA) for most diagnostic purposes, since the former offers a similar potential in sensitivity with increased versatility to a wider group of scientists (Aydin, 2015). ELISAs provide highly sensitive and precise methods for the estimation of biological parameters, with the added advantage that they can handle large numbers of samples that may then be analysed rapidly (Engvall, 2010). However, before they can be used, they must first be validated (see chapter 2).

Ideally, species-specific assays should be used because of the widespread species variation seen within the APP group, but there is only limited availability of cross-reactive reagents (Eckersall et al., 1991; Fransson et al., 2007). Some commercial ELISA kits are available to measure specific acute phase proteins, but most of them are expensive and lack automation (Cray et al., 2009). Nevertheless, measuring APP by ELISA is the best method for assessing large number of samples simultaneously (Aydin, 2015). For chickens, ELISAs have recently become available for SAA, CP and AGP, whilst, OVT recently redial immunodiffusion (RID) assay has developed (O'Reilly, 2016).

1.10 Non-invasive monitoring of APR induced pyrexia

Thermal, or infrared energy is the part of electromagnetic radiation that an observer detects as radiated heat. Infrared thermography (IRT) is a non-invasive method for

measuring radiated heat emitted by the skin, reflecting subcutaneous circulation and metabolism (Jones and Plassmann, 2002). Infrared thermography allows the visualisation and measurement of body surface temperature differences that occur during an inflammatory response and has been used in medical practice since the 1950s. In veterinary medicine IRT has been studied as a means of detecting clinical mastitis in dairy cows (Berry et al. 2003; Hovinen et al., 2008), the early onset of respiratory disease in calves (Schaefer et al., 2007), back pain in horses (Fonseca et al., 2006), hoof disorders in cows (Nikkhah et al., 2005), viral diarrhoea of calves (Schaefer et al., 2004), and the effect of milking on teat tissue (Paulrud et al., 2005). IRT has also been used to measures the severity of acute stress in chickens. This resulted in a rapid and short-term drop in temperature of the comb and wattle in laying hens, caused by vasoconstriction (Herborn et al., 2015). This is quite distinct from the sustained increase in body surface temperature that one would expect with Pyrexia. This technique has been utilised to detect of subclinical cases of leg pathology (bumble foot) in laying hens (Wilcox et al., 2009). In calves, IRT detected the onset of infection several days to 1 week before clinical score or serum concentration of APP indicated illness in the infected calves (Schaefer et al., 2004).

1.11 Models used in this thesis to stimulate and investigate the APR in chickens

The stimulants used in this thesis to investigate the APR in chickens and consequent APP production depend on many stimuli were i) vaccination using a Newcastle disease and Infectious Bronchitis (N/B) Live, freeze-dried virus vaccine; ii) an experimental challenge with Poultry red Mite (PRM); (iii) an LPS challenge experiment with an *E. coli* LPS. Justification for using these model systems is provided in the following paragraphs.

1.12 Vaccination and APP

The process of vaccination is an essential tool for optimizing health, welfare, and productivity of livestock. Trigger APP occurs as a normal result of vaccination in cattle (Stokka et al., 1994). Many factors could effect APR in terms of vaccination such as dose of the vaccine, presence and type of adjuvant, type and strain of pathogen utilised in the vaccine, consequently APP and rectal temperature were variable (Hernandez-Caravaca et al., 2017). The vaccines that produced the greatest

increase in rectal temperature were also those that produced the greatest magnitudes of increase in specific APP. Consequently, that has direct negative association with the average daily weight gain and final weight obtained in groups (Hernandez-Caravaca et al., 2017). Hernandez-Caravaca et al., (2017) have indicated that the preferred vaccine would be the one that produces the smallest increase in levels APP and rectal temperature. Responses of APP to vaccine stimuli have been investigated in other species; for instance, in horses (Andersen et al., 2012) vaccination against tetanus and influenza induced a prominent APR, with increased white blood cell count and APP (SAA and fibrinogen), decreased serum iron concentrations and an associated alteration in liver metabolism and skeletal muscles catabolism. Thus, Andersen at al. (2012) have advised horse owners to have a time for convalescence after vaccination. In sheep, routine vaccination of lambs with a multivalent clostridial vaccine with a *Pasteurella* component (Heptavac P™) showed that the APP responses caused an increase in the serum concentration of both haptoglobin (Hp) and SAA post vaccination. Interestingly, it has also been shown that the level of SAA in newly born lambs is influenced by maternal undernutrition prior to parturition, which subsequently is associated with a weakness in the innate immune system of the offspring (Eckersall et al., 2008). These authors concluded that maternal undernutrition can have detrimental consequences for offspring, in this case causing a reduction in an important mechanism of the innate immune system. Similarly, in growing beef calves, administration of commercially available *Mannheimia haemolytica* and Clostridium vaccines, have been associated with a significant increase of CP, SAA and haptoglobin (Arthington et al., 2013). These various studies have all reported an APR to a variety of vaccines and therefore suggest the possibility that monitoring the APP may be a means to determine the efficacy of a vaccine in stimulating the innate immune system and as such could be a tool of value in vaccine development. So, a vaccine that results in production of a low levels of APP is preferable (Hernandez-Caravaca et al.,2017), because there will be less effect on the production performance (Arthington et al., 2013).

Vaccination is a key strategy to mitigate the problem of infectious disease in chickens and immediate responses to vaccine components and related mediators such as Freund's complete adjuvant are known stimulants of the APP response (Eckersall et al., 2008; Koppenol et al., 2015). However, there is no previous study monitoring APP after vaccination with live attenuated viral vaccines in chickens.

Vaccines are used in the poultry industry to induce specific protection in chicks that either prevents clinical disease or at least greatly reduces pathogen replication, so limiting the spread of disease. In this study APP response to a vaccine containing Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) will be studied. IBV is an acute and highly contagious viral disease causing severe economic losses for those involved in the chicken industry (Cavanagh, 2007). Morbidity rate can reach 100% and mortality may reach 80% depending on the virulence of the strain (Awad et al., 2014). Newcastle disease, according to (Czegledi et al., 2006) is one of the top-ranking diseases that affect the poultry industry in terms of economic loss due to high morbidity and mortality rates of up to 100% (Czegledi et al., 2006). According to these authors "the entire globe is burdened by the threat of Newcastle disease except coastal and oceanic zones".

A number of studies have investigated AGP in virus-infected chicks (Nazifi et al., 2010; Xie et al., 2002b; Sylte and Suarez, 2012). Both IBV and infectious laryngotracheitis virus (ILTV) increased plasma levels of AGP, with higher levels reported in the IBV than ILTV infected chickens (Nakamura et al., 1996). It has been reported that AGP concentrations increased in chickens that had Gumboro disease and the levels increased with increasing severity of the clinical signs of the infection (Inoue et al., 1997). AGP concentration was also increased in birds challenged with a low pathogenicity strain of avian influenza virus (LPAI) (Sylte and Suarez, 2012), and interestingly, the level of AGP in the vaccinated birds was less than in the unvaccinated groups (Sylte and Suarez, 2012), this is because vaccination provides specific protective antibodies, which reduce the severity of the disease. There is a strong relationship between the severity of a disease and the response level of APP (Nazifi et al., 2010). Consequently, after challenge the level of APP will be less than that of unvaccinated groups (Martinez-Subiela et al., 2002). (see also chapter 3).

1.13 Poultry Red Mite infestation

Poultry red mite (PRM) *Dermanyssus gallinae* is one of the significant threats to the egg production sector of the poultry industry in many parts of the world, including Europe, the USA, Japan and China (Chauve, 1998; Sparagano et al., 2009). In the United Kingdom, between 60 and 85% of commercial egg-laying facilities may be infested with *D. gallinae* (Sparagano et al., 2014; Fiddes et al., 2005). It is classified as a hematophagous ectoparasite of poultry and wild birds (Brannstrom et al., 2008;

Kristofik et al., 1996). It has been reported that females of this species need blood permanently throughout their life cycle, while males feed on blood occasionally (Valiente et al., 2005). It has been estimated that PRM infestation could increase mortality by between 4 and 50% and reduce egg production by as much as 20% (Cosoroaba, 2001). The annual economic loss for the EU egg industry as a result of PRM infestation is estimated to be around €130 million (Harrington et al., 2007).

PRM resistance to some of the acaricide products including carbamates and pyrethroids in the UK have been widely reported throughout Europe viz; UK (Fiddes et al., 2005), Sweden (Nordenfors et al., 2001) France (Beugnet et al., 1997) and Italy (Marangi et al., 2009). These authors pinpointed misuse as the main reason for this development. Resistance to acaricides, changes in the legal use of pesticides, the number of acaricides licenced for PRM treatment is severely limited in the European Union (Roy et al., 2009; Flochlay et al., 2017). Vaccination with arthropod protein antigens such as those from the salivary gland or midgut, has been shown to induce significant immunity against PRM infestation (Titus et al., 2006). Immunisation of birds with whole PRM antigens has shown significantly higher IgY titres in immunised versus control groups (Harrington et al., 2007). The APR to these vaccines however has not been studied and could yield important information in terms of assessing vaccine efficacy under challenge conditions. The APR associated with a PRM infestation is the subject of Chapter 4 of this thesis as a prelude to studying the response following the administration to vaccination.

1.14 *Escherichia coli* endotoxin challenge

The historical aspects of the role of endotoxins in bacterial pathogenesis (Beutler and Rietschel, 2003) and their chemical characterization as lipopolysaccharide (LPS) (Raetz, et al., 2007; Raetz and Whitfield, 2002) have been the subject of some comprehensive reviews. The response from the host immune system depends on both the severity of infection and the particular structure of LPS of the invading bacteria. High concentrations of LPS can induce fever, increase heart rate, and lead to septic shock and death (Parrillo, 1993). In dairy cow, LPS can induce an inflammatory response, increased body temperature, decrease milk production, reduce milk quality, decreased feed intake and, and altered the profiles of milk fat and milk protein in lactating (Ning et al., 2018). It has been concluded in other animals that single-dose *Escherichia coli* (*E. coli)* endotoxin challenge activates the APR and that

this model can be used for examining the pathophysiology of inflammation and infection (Orro et al., 2004). The LPS triggered immune activation leads to the production of inflammatory mediators (cytokines and chemokines) (Haudek et al., 2003; Skarnes et al., 1981), which influence the blood protein profile, including acute phase proteins (APP) (Salomao et al., 2012). Since APP have different stimulation patterns, monitoring of them can provide more information on the ongoing APR of the host and thus may provide a useful tool in veterinary medical science (Orro et al., 2004).

Plasma concentrations of AGP increased significantly 12 hs after *E. coli* endotoxin injection in one study (Takahashi et al., 1998), but other studies found the levels increased later, reaching a peak 48 h post infection (Nakamura et al., 1998). Induction of infection by *Salmonella* Typhimurium LPS and turpentine oil resulted in significantly increased levels, peaking four-fold higher than the starting level at 24 h post-infection (Adler et al., 2001). Trace minerals in blood were also significantly altered throughout the APR. These changes included increased copper and decreased zinc and iron (Johnson et al., 1993). The zinc and iron changes seem to be a result of hepatic sequestering and increases in the level of hepatic metallothionein, another chicken APP (Klasing and Johnstone, 1991). Copper and iron levels are also linked to CP and OVT respectively (Martinez-Subiela et al., 2007; Aguilera et al., 2003). Chickens infected with secondary bacterial infection that accompanied coccidiosis, showed an increase in CP, as well as increased levels of zinc, copper and iron in serum (Richards and Augustine, 1988).

1.15 Hypothesis

In poultry there is an increasing emphasis on the identification of biomarkers for enhancing immunity and disease resistance to confirm both diagnosis and prognosis of diseases. Viral, bacterial and parasite pathogens remain a threat to the poultry industry and countermeasures to prevent and control these are needed due to prevent production losses.

Thus, the current study was designed to test the hypothesis that the innate immune system can be triggered by different pathogens, consequently altering the level of APP. Therefore, monitoring the levels of APP can be employed to estimate the vaccine efficacy in stimulating the immune system. On the other hand, challenge

with PRM and LPS might trigger different APP expression profile at different time points that might help in early detection and diagonosis of the onset of the challenge. The hypothesis of each chapter have been described in detail in the relevant chapter.

1.16 Aims of Thesis

The overall aim of the current thesis was to identify blood components associated with the acute phase response that could help with combating several much-needed facets of disease control such as:

1. Validate tests for APP
2. Early detection and diagnosis of diseases in chickens
3. That some of the APP might also prove helpful tools for assessing the usefulness of a vaccine in terms of its likely immunogenicity
4. Show that some of the APP we studied might be useful tools for genetic selection against *E. coli* and PRM.

The main candidates identified to study were four types of APP (SAA, AGP, OVT and CP). The first aim of the thesis was to validate the species-specific ELISA assays that are now commercially available for SAA, AGP and CP in chicken plasma. This forms the basis of chapter 2 of the thesis.

Whereas,the aim of Chapter 3 was to investigate the APP response in specific pathogen free (SPF) layer chicks for 6 days following the intraocular administration of a combined Newcastle disease and Infectious Bronchitis (N/B) Live, freeze-dried virus vaccine. The H/L ratio of vaccinated and unvaccinated birds was also assessed in this study as it is considered to be part of the APR. The H/L ratio is routinely used as a measure of stress in poultry.

Investigatation of the APP response over the time course of an experimental PRM infestation in laying hens was the aim of Chapter 4. In addition to evaluate if a PRM infestation significantly altered the serum protein profile of laying hens. As this experiment began during the rearing phase, another aim was to determine if a PRM challenge at the onset of lay, resulted in any unanticipated changes in the major serum proteins.

The aim of the study presented in Chapter 5 was to measure and compare the plasma levels of corticosterone, the H/L ratio and the systemic plasma levels of three APP (AGP, SAA and OVT) over a time course of 72 hours of experimental challenge with LPS *E.coli*. To the best of the author knowledge this is the first time that the APR response has been studied over the time course of the entire febrile response in chickens.

I also assessed if thermography could be used as a non-invasive tool to detect one component of the acute phase response by monitoring surface body temperatures changes. Assessing if these changes correlated with recorded core body temperature with the onset of pyrexia in LPS challenged broiler chickens was the aim of Chapter 6. Fever is an essential component of the acute phase response, so monitoring it by thermography as a non-invasive tool could provide a novile method for monitoring APR that can be cheaper and faster than the other invasive tools. .

Finally, Chapter 7, a general discussion chapter, summarises the work of the whole thesis, how well these aims were fulfilled and makes recommendations for future work.

Chapter 2 Validation of ELISA kits

2.1 Introduction

Enzyme immunoassay EIA and enzyme-linked immunosorbent assay (ELISA) are both widely used as diagnostic tools in medicine and as quality control measures in various industries; they are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given sample (Gan and Patel, 2013). Enzyme linked immunosorbent assay (ELISA) is a well-established biochemical method that has been widely used in various biomedical applications to determine binding sensitivity, or to specifically detect bio-analyte concentrations in biological fluids (Aydin, 2015). ELISA utilizes enzyme-labelled antigens and antibodies to detect the biological molecules. The most commonly used enzymes are alkaline phosphatase and glucose oxidase. The antigen in fluid phase is immobilized, usually in 96-well microtiter plates allowing the antigen to bind to its specific antibody, which is subsequently detected by a secondary, enzyme coupled antibody. A chromogenic substrate for the enzyme yields a visible colour change or fluorescence, indicating the presence of antigen (Gan and Patel, 2013). Quantitative or qualitative measures can be assessed based on such colorimetric readings. Fluorogenic substrates have higher sensitivity and can accurately measure levels of antigen concentrations in the sample (Gan and Patel, 2013; Engvall, 2010).

The fact that proteins (including antibodies), and carbohydrates can be passively attached to plastics has been exploited in most applications of ELISA. Since one of the components is attached to a solid phase by passive absorption, subsequent reagents can be added, and after a period of incubation, unreacted material can be simply washed away. Such assays are termed Heterogeneous ELISAs. Various types of ELISAs have been employed with modification to the basic steps, the key step in the ELISA assay is the direct or indirect detection of antigen by adhering or immobilizing the antigen or antigen-specific capture antibody, respectively, directly onto the well surface. For sensitive and robust measurements, the antigen can be specifically selected out from a sample of mixed antigens via a “capture” antibody. The antigen is thus “sandwiched” between such capture antibody and a detection antibody. If the antigen to be measured is small in size or has only one epitope for antibody binding, a competitive method is used in which either the antigen is labelled and competes for the unlabelled antigen–antibody complex formation, or the antibody is labelled and competes for the bound antigen and antigen in the

sample. Each of these modified techniques of ELISA can be used for a qualitative and quantitative purpose. Thereafter ELISA assay has been used widely by different researchers to estimate various biological fluids (Car lsson et al., 1972; Holmgren and Svennerholm, 1973; Ljungstrom et al., 1974). ELISA is the method which has found different fields of application and has grown over time to become a routinely used technique in research and diagnosis laboratories around the world (Aydin, 2015). The commercial availability of ELISA kits in clinical investigations is fundamentally important and has been manifested in a diverse range of applications (Lequin, 2005). Before an ELISA kit can be routinely used however it must first be validated by assessing its precision, accuracy and limit of detection. The reproducibility of the results and their interpretation is dependent on this (Lumsden, 2000).

So far APP have been measured in chickens using non-specific ELISAs (Nazifi et al., 2010; Asasi et al.,2013) or radial immunodiffusion assays (Holt and Gast, 2002; Inoue et al., 1997; Nakamura et al., 1996,). In this chapter three commercially available chicken specific ELISAs for α1- acid glycoprotein (AGP), serum amyloid A (SAA) and ceruloplasmin (CP) are validated using of samples known provenance (classified as high, medium and low APP). Within my own laboratory it was important to assess the ability of these assays to differentiate between different levels of APP responses, prior to application in the rest of this thesis.

2.2 Materials and methods.

The serum samples used in this validation study were obtained from a previously reported study coming from chickens that had been classified as exhibiting a high, medium or low APP response (O'Reilly, 2016). These samples were used as quality control for determining inter-assay and intra CV% and also for accuracy.

2.2.1 ELISAs for AGP, CP and SAA

ELISA kits for the AGP-5, CP-5 and SAA-5, were purchased from Life Diagnostics Inc, (West Chester, USA), and unless stated were utilised as per the manufacturer instruction. The AGP (purified from chicken plasma) and SAA (synthetic SAA polypeptide was used to prepare standard) standards provided with the kit were diluted 7 times from 150ng/ml to 2.34ng/ml for AGP and from 15µg/ml to 0.23µg/ml for SAA. For CP (purified from chicken plasma), 6 serial dilutions were made from 50ng/ml to 1.5ng/ml.

For the AGP and CP ELISAs, 100µl of diluted serum samples (final dilution 1: 10,000 for AGP, 1: 40,000 for CP respectively) and standards were then dispensed into duplicate wells of each 96-well microtiter plate. Each plate was then incubated on an orbital microplate shaker at 150 revolutions per minute (rpm) at room temperature (RT) for 45 min.

For SAA, 50µl of sera was first incubated in a heat block at 60^{o}C for one hour, to dissociate SAA from lipoproteins. Following heat treatment, the serum was diluted 1:20. 100µl of the diluted sample (or standard) was then dispensed into duplicate wells of the 96-well microtiter plate coated with anti-SAA, together with a detector antibody (two peptide epitopes were identified as they were immunogenic and exposed in the native protein, peptides were conjecated to keyhole limpet hemocyanin (KLH) and antibody (rabbit) made agiest each then antibodies were purifies on approperate peptide-agarose column and conjugated to HRP (100µl) for one hour. As a result, SAA molecules become sandwiched between the immobilisation and detection antibodies.

For all 3 APP the contents of the wells in each plate were then discarded, and the wells were washed five times each using wash buffer provided with the kit. For AGP and CP, after ensuring all residual droplets in the wells were removed by striking plates onto absorbent paper, and, 100µl of the secondary antibody-HRP conjugate were prepared by immunization rabbit with pure protein and then antibodies have been purified by properate angen-agarose column. was then dispensed into each well before incubating for a second time at RT for 45 min and repeating the washing step as above.

For all assays 100µl of TMB reagent (3,3′,5,5′-Tetramethylbenzidine, HRP substrate) was then dispensed into each well and blue colour development was allowed to proceed for 20 min by places the plate on a shaker at RT. The reaction was then stopped by adding 100µl of 1M HCl into each well. The absorbance was read using a FLUOstar Optima plate reader at 450 nm within 15 min of stopping the reaction. A four-parameter logistic curve (4PL) was then chosen from the Optima software to generate each standard curve.

2.2.2 Precision and detection limit

The precision of each ELISA kit was determined as intra assay percentage of coefficient of variation (intra %CV), by comparing 40 sera samples in duplicate within same plate, and the inter assay %CV, comparing two quality control samples (high and low) on 5 different plates.

$$\%CV = \frac{\text{Standard deviation}}{\text{Mean}} X100$$

The %CV was considered acceptable if values were <10% for intra-assay imprecision, and <20% for inter-assay imprecision, as described in previous studies (Jacobson and Romatowski, 1996; Kjelgaard-Hansen and Jensen, 2010). The detection limit was assessed by using 4 blanks made from the diluent supplied in each ELISA kit. The detection limit was calculated as the mean + 3 standard deviations of the blank samples at the 99.5% confidence level (Kjelgaard-Hansen and Jensen, 2010).

2.2.3 Accuracy

The accuracy of each assay was determined by comparing serial dilutions of samples (n=4) with assay buffer, the high APP content samples and were assessed for parallelism to the standard curve. GraphPad Prism software was performed to generate the line graph of the correlation between measured and expected values to determine the linearity under dilution was assessed by using a least squared regression test.

2.2.4 Specificity

A Western blot assay was used to determine the ELISA kits specificity to determine the cross reactivity of the antisera (high, medium and low APP samples) used in the ELISAs (see section 1.2.6.3). The protein content of selected serum samples was determined prior to the SDS-electrophoresis and Western blot. The total protein concentration of each serum sample (high, and low APP) were determined by the Bicinchoninic acid assay (BCA) (Uptima from interchim Montluçon, France) with bovine serum albumin (Seifi, et al.) (Sigma Aldrich, Dorset, UK) used as the standard where diluted by dH_2O to make 8 different concentrations from S1 (2mg/ml) to S8 (0.25 mg/ml).

Serum samples were diluted 1:10 using millipore dH_2O and then 10 µl of each standard and sample were transferred in duplicates into a 96-well plate (Falcon # 351172). The BCA assay reagent was then added (200 µl/well) and mixed carefully on a plat shaker for ~20 seconds. The microplate was incubated at 37°C for 30 min. ELISA reader (FLUOstar OPTIMA spectrophotometer at 595 nm (BMG Labtech, Aylesbury, UK). was used at 562 nm wavelength to determine the optical density of the tested samples. The protein concentration was determined using a standard curve that was plotted by the spectrophotometer according to the BSA standards (absorbance versus concentration in mg/ml) and taking the dilution factor into consideration.

Note: Chapter 4 in this thesis, the total protein concentration was measured using the Bradford assay system. For this assay standard (Seifi, Alian Samakkhah and Absalan Fard) preparation and samples dilution were as above. Bradford reagent (appendix B section 2.1) (Sigma #SLBC4590V 250 µl) was added to each well of a 96

plate and then 5µl of dilute samples were added. The mixture was then mixed well by a plate shaker for ~20 seconds then incubated at RT for 15 minutes. The results were read at optical density 595 nm by Optima reader.

2.2.5 SDS PAGE one-dimension electrophoresis (1DE)

10µl of pooled serum sample from the high, medium and low APP serum samples (4 mg/ml), was diluted using milli Q water and then Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 25 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) bromophenol Blue) (1 :1 with sample) to be a final concentration 2mg/ml. This mixture was then heated at 95^{o}C for 5 min. Samples were then loaded into the wells of a BioRad Criterion XT Bis-Tris precast 18 well gel (BioRad #345-0124). A molecular weight marker was then added to the first well and voltage of 300V applied for approximately 20 minutes (before the bands reached the edge of the gel) in MOPS running buffer (BioRad #161-0788) (appendix B section 2.2).

Note: Samples for SAA western blot were heated 60^{o}C for one hour, as recommended in the procedure of the ELISA instructions. Then these samples were diluted to be 6mg/ml, as final concentration and 10ul were loaded in 1D gel, instead of the 2mg/ml for other western blots.

2.2.6 Western Blot

1D gels was removed from the cassette, and placed directly onto a nitrocellulose paper (NCP 0.45um) (Bio-Rad laboratories Inc., Germany, 162-0112), which was soaked in transfer buffer (appendix B section 2.4) (6.06 g Tris-HCl, 28.8 g Glycine in 1600 ml milli *Q* water and 400 ml methanol) to wet the NCP and avoid damage to the gel. The gel and NCP was placed between blotting paper and foam pads on both sides, and sealed within a cassette holder and then put into a tank with ice packs. Criterion blotter (Bio-Rad 170-4070) was used in this method to blot protein bands. Transfer buffer was then added to the blotting tank (Bio-Rad Criterion, USA), and run at 70V for 1 h. Then the NCP was blocked overnight using 1% (w/v) non-fat milk protein in TBS buffer. The next day, the NCP paper was washed three times with 0.1% tween-20 in TTBS buffer (TTBS) (appendix B section 2.3) . The primary antibody (Rabbit anti-chicken SAA, Lot C-D 22148, Rabbit anti-chicken CP, Lot C-H 0614A or Rabbit anti-chicken AGP, Lot C-L 23088) which were the antibodies used in the ELISA

were diluted 1:1000 and 1:5000 for SAA. Whilst 1: 10,000 and 1: 20,000for AGP and CP, in TTBS with 5% non-fat milk, then poured over the NCP paper, and placed on the rocker for 1 h at room temperature. The NCP paper was washed three times, and then the paper was incubated with the secondary antibody (donkey-anti rabbit IgG (diluted at 1: 10,000 in 5% non-fat milk), for 1 h by placing on the rocker at RT. A TMB chromogenic substrate (Lot# RB 226056A, Thermo Scientific, UK) solution was then applied for 1-3 min to allow colour development of the AGP, CP or SAA reactive bands. Each Western blot was then scanned using Umax powerlook III (Hamrick software, USA).

2.3 Results

2.3.1 The standard Curve

The concentration of chicken AGP, SAA and CP standards ranged from 150- 0 ng/ml, 5- 0.23 µg/L, and 50-1.5 ng/ml respectively, and gave the 4-parameter line curve fit on the log-linear scale as illustrated in Figure 2.1.

2.3.2 Assay precision

Overall the precision of each assay was acceptable. The intra assay % CVs were lowest for CP (4.3%), followed by SAA (5%) and AGP (6%) (Table 2.1 – 2.3). A similar result was observed for the inter assay % CVs: 9.14%-13.55% for CP; and 10-14% for SAA; 23-25% for AGP. The limit of detection for the AGP, SAA and CP were 0.05 g/L, 0.21 µg/L and 0.06 g/L respectively.

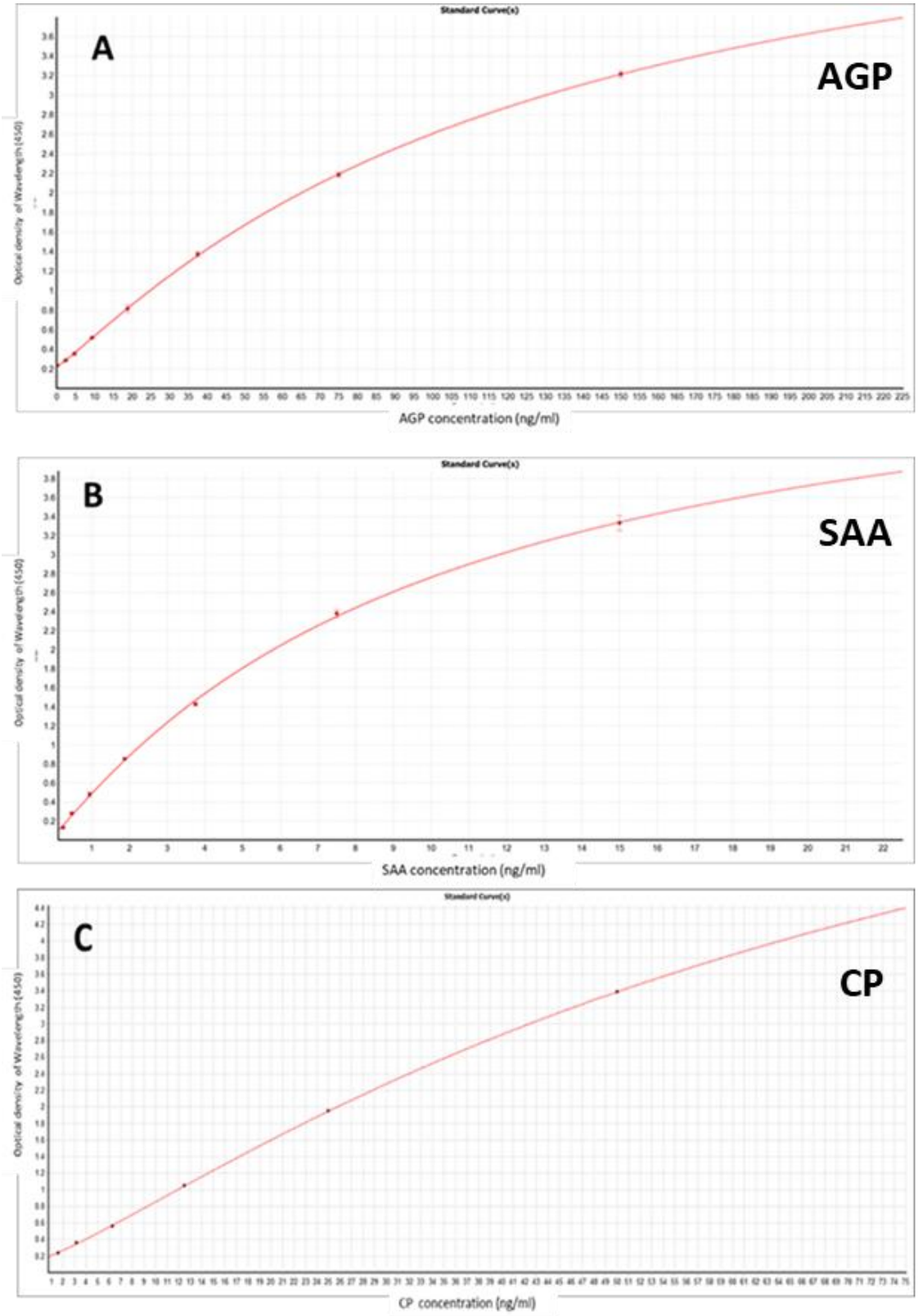

Figure 2.1 Standard curve of AGP, SAA and CP.
4 parameter fit standard calibration curves for A) AGP, B) SAA and C) CP. 7 different concentration points were used for AGP (150 - 2.34 ng/ml) and SAA 1(5- 0.23 ug/L), and 6 points for CP (50-1.5 ng/ml).

Table 2.1 Coefficient of Variation (CVs) for the AGP assay.
Intra-assay CVs were calculated (n=40) to determine the within plate variation. Two samples were used: a high and low-quality control. The inter-assay CV was calculated from running two samples (a high and low) over five assay runs. The values of which were then used to calculate a CV to determine repeatability.

Intra – assay CV	Mean (n=40)	SD	CV	Limit of Detection
	0.45 g/L	1.17	2.741%	0.05 g/L
Inter- assay CV of AGP	**QC - low**	**QC – high**		
	0.50	1.19		
	0.37	1.67		
	0.43	1.20		
	0.49	1.22		
	0.33	1.24		
Mean	0.42	1.30		
SD	6.55	0.18		
CV	15.3%	14.2%		

Table 2.2 Coefficient of Variantion (CVs) for the SAA assay.
Intra-assay CVs were calculated the SD of the mean (n=40) to determine the within plate variation. Two samples were used: a high and low of quality control. The inter-assay CV was calculated from running two samples (a high and low) over five assay runs. The values of which were then used to calculate a CV to determine repeatability.

Intra – assay CV	Mean (n=40)	SD	CV	Limit of Detection
	25.96 µg/L	1.88	3.06 %	0.21 µg/L
Inter- assay CV of SAA	**QC - low**	**QC – high**		
	7.11	116.46		
	10.55	102.29		
	6.93	159.28		
	11.92	155.13		
	11.80	121.3		
Mean	9.66	130.89		
SD	2.21	22.41		
CV	22.8%	17.1%		

Table 2.3 Coefficient of Variantion (CVs) for the CP assay.
Intra-assay CVs were calculated the SD of the mean (n=40) to determine the within plate variation. Two samples were used: a high and low of quality control. The inter-assay CV was calculated from running two samples (a high and low) over five assay runs. The values of which were then used to calculate a CV to determine repeatability.

Intra - assay CV	Mean (n=40)	SD	CV	Limit of Detection
	1.05 g/L	0.05	4.30%	0.06 g/L
Inter- assay CV of CP	**QC - low**	**QC - high**		
	0.320	2.53		
	0.392	2.03		
	0.321	2.60		
	0.439	2.37		
	0.356	2.35		
Mean	0.365	2.38		
SD	0.04	0.21		
CV	13.55%	9.14%		

2.3.3 Accuracy of the AGP, SAA and CP ELISAs

The results show that dilution of 4 serum samples with high concentrations of the different APP resulted in high correlation coefficients for AGP, SAA and CP were r^2= 0.986, 0.984 and 0.989 respectively with P<0.001 for each test (Figure 2.2 A-C).

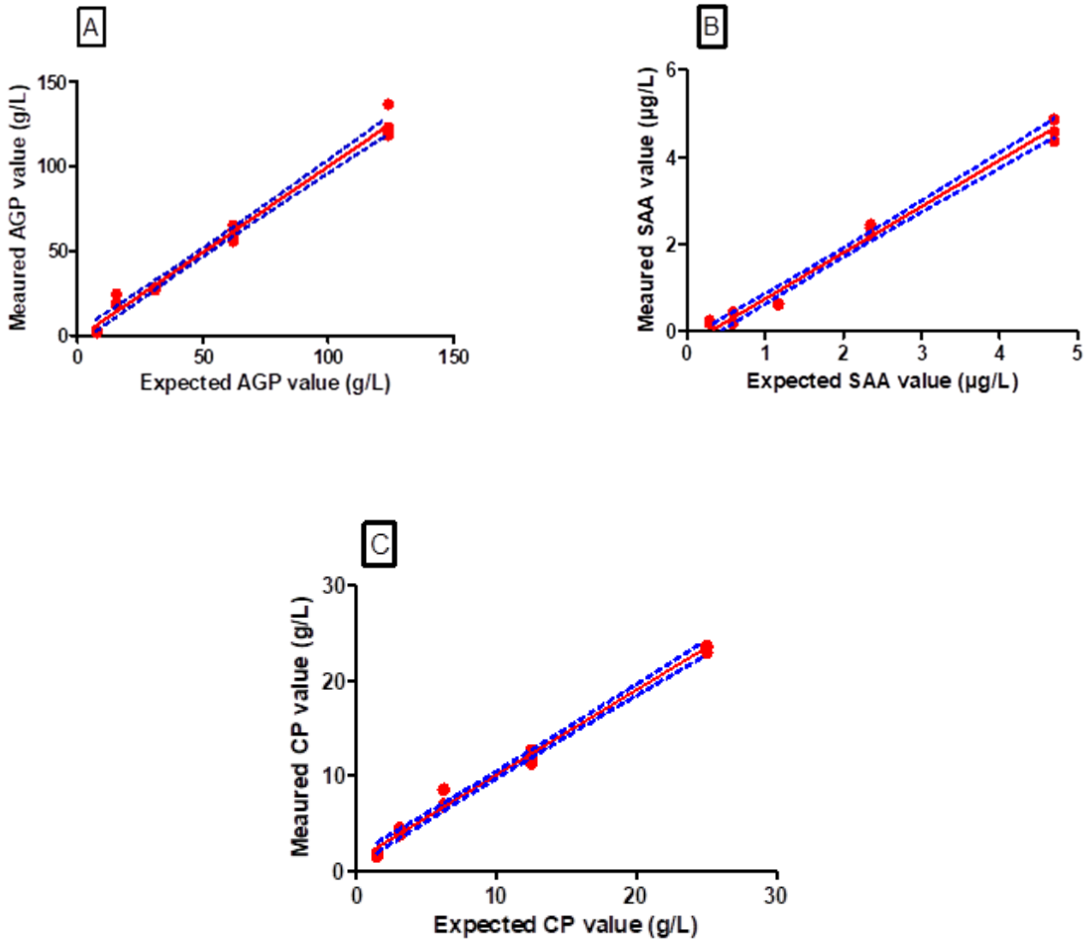

Figure 2.2 Linearity under dilution.
Each data point indicates the mean of a four measurement. The solid lines indicate the linear fit, the dotted lines represent the 95% Confidence Interval (CI). The dashed lines represent the 95% Prediction Interval. A) AGP linearity r^2=0.986, B) SAA linearity r^2=0.986 and C) CP linearity r^2=0.989.

2.3.4 Specificity

Specificity of the SAA, AGP and CP, assays were tested by Western blot (Section 1.2.6.3). Immunoblot bands appeared with the utilisation of primary antibody of SAA, AGP and CP to react with the separated chicken serum proteins (Figure 2.3A, 2.4A and 2.5A), but as expected, no bands were observed when the primary antibody was omitted (Figures 2.3B, 2.4B and 2.5B).

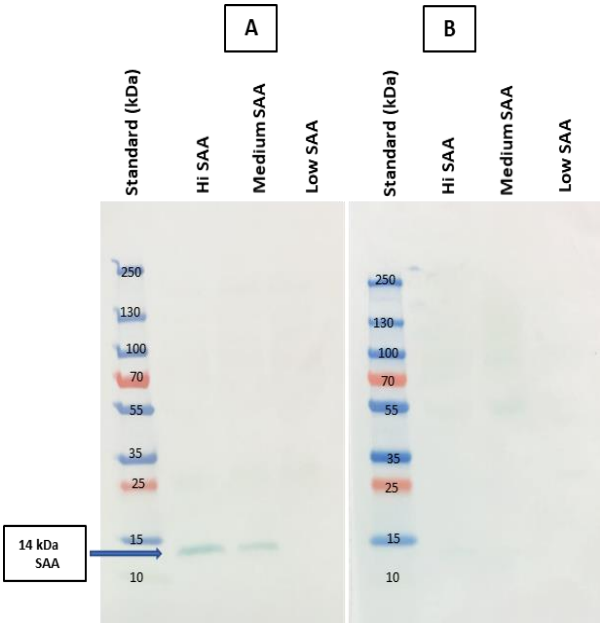

Figure 2.3 Western blot of chicken serum SAA samples.
(A) the bands of SAA in three pooled samples from birds with high, three pooled samples medium and three pooled samples low concentration of APP, rabbit anti-chicken antibody diluted 1: 5000, Where the primary antibody has been omitted (B) serum proteins were separated by with 4-12% Criterion TM Bis-Tris precast polyachramide gel (BioRad#345-0124) prior to western blotting.

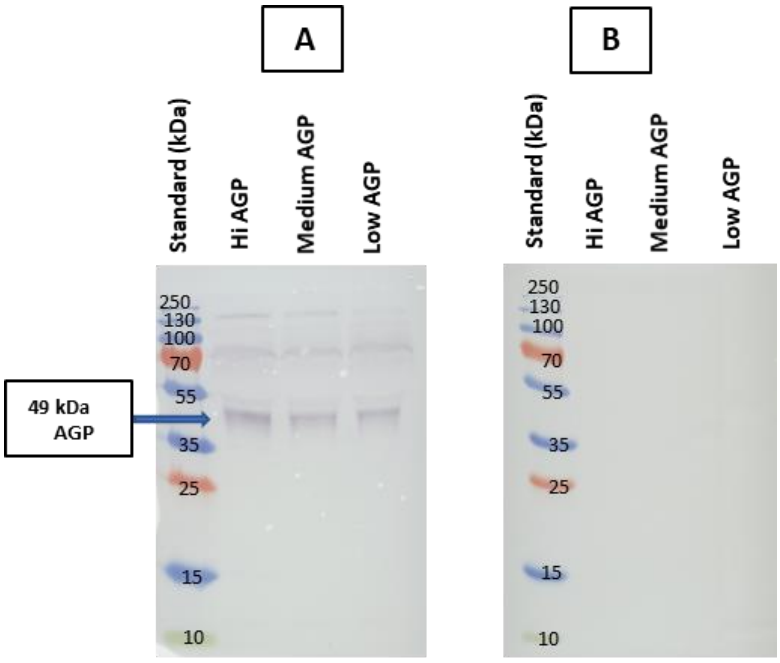

Figure 2.4 Western blot of chicken serum AGP samples.
The bands were developed from AGP three pooled samples from birds with high (lane 2), three pooled samples medium (lane 3), and three pooled samples low (lane 4) concentration of APP, where the primary antibody (dilution 1:10 000) has been included (A) or omitted (B). serum proteins were separated by with 4-12% Criterion TM Bis-Tris precast polyachramide gel (BioRad#345-0124) prior to western blotting.

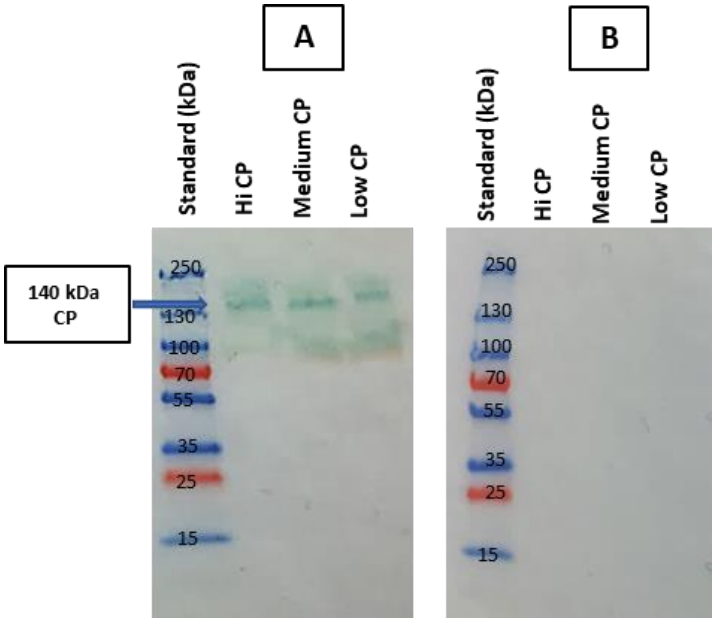

Figure 2.5 Western blot of chicken serum CP samples.
(A) the bands of CP in three pooled samples from birds with high, three pooled samples medium and three pooled samples low concentration of APP, rabbit anti-chicken antibody diluted 1: 20,000, Where the primary antibody has been omitted (B) serum proteins were separated by with 4-12% Criterion TM Bis-Tris precast polyachramide gel (BioRad#345-0124) prior to western blotting.

2.4 Discussion

The inter CV results for AGP and CP show low percentages which indicate high precision for these assays. For SAA the inter CV was 22.8% with quality control samples with a known low concentration of SAA (Table 2.2). Other researchers have performed studies to evaluate validity of ELISA assays for APP, and have accepted high CV measurements (39.4% inter essay CV) for CRP and SAA essays (Kjelgaard-Hansen et al., 2003). In addition, Martinez and co-workers, have reported that a 26% inter essay CV is reasonable (Martinez-Subiela et al., 2002). In general, CVs higher than 15% are not acceptable for the analytical point of view (Martinez-Subiela and Ceron, 2005), and such high CVs could lead to misinterpretation of the results (Eckersall et al., 1999) although in some situations these assays can be of value. The high imprecision could be minimised by performing all analyses in the same batch (Martinez-Subiela and Ceron, 2005). Furthermore, improvement of the assay precision could be achieved by using an automated assay system to avoid errors resulting from manual pipetting and dilutions. Finally, efficacy of the immunological assays could be improved by using a species specific calibrator (Tecles et al., 2007b). It is noteworthy to mention that a high CV found in APP assays, would not have clinical relevance because of the significant range and wide differences between healthy and pathological samples (Tecles et al., 2007a). Therefore, to confirm the specificity of the obtained results from the ELISA experiments, it was important to examine the specificity of the utilised antibodies using another experimental methodology such as western blot.

The current study revealed that western blot of chicken samples with the specific antibody of the rabbit anti-chicken CP developed bands at the expected molecular weight of 140 kDa (Figure 2.3), which was similar to other studies (Calabrese et al., 1988). AGP western blot with specific antibody of the rabbit anti chicken AGP showed development of bands at 49kDa. This finding was also in agreement with previous reports (Ceciliani and Pocacqua, 2007).

Western blots of chicken serum with anti-SAA antibody showed development of a band at 14kDa, which is similar but not identical to previous reports (Soler et al., 2013; Wang et al., 2002). The latter authors reported that SAA has a molecular weight of 12kDa. In the current study, a band did not develop in the blots unless the samples were heated prior to running the 1D gel. This was in line with the SAA ELISA

instructions which stated that samples had to be heated to 60^{o}C for one h. The importance of this step was to allow dissociation of the SAA from high-density lipoproteins (HDLs) in plasma (Wang et al., 2002). Furthermore, for SAA the concentration of the sample applied to the gel was increased to 6 mg/ml (Figure 2.3) as the 2mg/ml concentration resulted in the development of very fine bands.

The detection limit obtained for each assay allowed the determination of APP concentrations with low concentration enough to detect serum levels in all groups. These results would indicate that all APP methods studied were able to discriminate between samples with low and high APP concentrations and quantify the presence of variable levels in the chicken samples.

In conclusion, the performance of the chicken ELISA kits for measuring serum concentration of AGP, SAA and CP were acceptable for use and can give valid results in the chickens' samples, since they provide good accuracy, a suitably low detection limit and allow discrimination between different levels of APP in chicken samples. The ELISAs can be utilised in experimental studies and in clinical cases to evaluate the APP levels in healthy and pathological cases of chickens.

Chapter 3 Acute phase proteins (APP) and stress markers in the immediate response to a vaccination in specific pathogen free (SPF) layer chicks.

3.1 Introduction

There is increasing interest in APP in chickens as a physiological marker for health and welfare, including infection and, intriguingly in response to routine vaccination for both bacterial and viral pathogens (O'Reilly and Eckersall, 2014). Responses of APP in terms of vaccination stimuli have been investigated in other species; for instance, in horses (Andersen et al., 2012), sheep (Eckersall et al., 2008) and calves (Arthington et al., 2013). These previous studies reported an acute phase response to a variety of vaccines and therefore suggest the possibility that monitoring the APP may be a means to determine the efficacy of a vaccine in stimulating the innate immune system and as such could be a tool of value in vaccine development.

In chickens, alpha-1 acid glycoprotein (AGP) has been shown to respond as a moderate positive APP (Chamanza et al., 1999b; O'Reilly and Eckersall, 2014) following experimental infection. For example, in 3-week-old White Leghorn SPF chicks inoculated via the intraocular route with a highly virulent strain of Gumboro disease virus, there was a 4.6-fold increase in serum AGP concentration at 2 d post challenge. Whereas, inoculation with an attenuated strain of Gumboro disease virus lead to a 2.4 fold difference in AGP at d 2 post inoculation (Inoue et al., 1997). AGP levels have also been shown to increase significantly between 12 and 48 h post IV injection with lipopolysaccharide (LPS) from Escherichia coli in 3-week-old male broiler chickens (Takahashi et al., 1998). In commercial layer chickens inoculation with Mycoplasma gallisepticum vaccine caused a significant increase in serum AGP concentration at 1 d post vaccination (Peebles et al., 2014). In this study the vaccine was administered via the intraocular route in one group, and subcutaneous injection in another group. The concentration of AGP remained significantly higher in both the vaccinated groups irrespective of the mode of administration compared to the control group for up to 21 d post inoculation.

Serum amyloid A (SAA) is the only major positive APP found in chickens (Alasonyalilar et al., 2006; O'Reilly and Eckersall, 2014). SAA is described as being immunomodulatory, inhibiting pyrexia and down regulating pro-inflammatory events during an APR (Shainkinkestenbaum et al., 1991; Uhlar and Whitehead, 1999). Due to the lack of a commercial assay system however, there have been few reliable reports on this APP in chickens (Alasonyalilar et al., 2006, Nazifi et al., 2010, 2011) and none in relation to post vaccination (PV) stimulation.

Ceruloplasmin (CP) and ovotransferrin have both been described as minor/moderate positive APP in chickens. CP, as with most APP, is mainly synthesised in the liver, and this synthesis mainly occurs in the cytoplasm of hepatocytes (Floris et al., 2000). The main function of CP is to store and transfer copper within the body which gives it an important antioxidant role (Floris et al., 2000). There are no specific reports on how this APP responds in relation to vaccination but Butler and co-workers, (1972) and others have reported a 2-5 fold increase in the CP level following injection with *E. coli* LPS which reached a maximum at 24 h post injection. Blood CP have also been reported to change in response to various infectious agents such as *Eimeria tenella* (Richards and Augustine, 1988; Georgieva et al.,2010) and combined *E. coli* and *E. tenella* infection (Georgieva et al., 2010).

Ovotransferrin (OVT) is synthesised in hepatocytes but in laying hens it is also produced in the cells of the oviduct under the influence of oestrogen (Hallquist and Klasing, 1994). The main biological function of OVT is to transport minerals like iron in the body (Hallquist and Klasing, 1994; Xie et al., 2002a). Consequently, OVT can demonstrate an antibacterial activity by controlling the transport of iron, which is an essential element for bacterial growth (Xie et al., 2002b). Blood OVT levels have been shown to change in chickens in response to infectious diseases, metabolic disease and autoimmune disease (Rath et al., 2009; Xie et al., 2002b) but changes appear to only be evident following pathogenic challenge (O'Reilly and Eckersall, 2014). This APP is therefore unlikely to change in response to mild stimulation such as routine vaccination, but this has not yet been determined.

This chapter describes an experiment where the APP response was measured and monitored for 6 days in SPF layer chicks following the intraocular administration of a combined Newcastle disease and Infectious Bronchitis (N/B) live, freeze-dried virus vaccine. For AGP, SAA and CP the commercially available ELISA assays that were validated in chapter 2 were applied and used. Such an assay system is not available for chickens OVT, so this APP was measured using a radial immunodiffusion assay (O'Reilly, 2016).

Changes in white blood cell count, especially in heterophil/lymphocyte (H/L) ratio, have been used as a measure of stress in chickens (Gross and Siegel, 1983; Shini et al., 2008). The H/L ratio of birds can be affected by health disturbance or stress

(Crowther, 2009), including transport stress (Huff et al., 2005; Matur et al., 2016), possibly due to the transition of leukocytes from the marginal pool to peripheral circulation (Duncan, 1987). In this study, the H/L ratio was measured to establish if there was a link between the stress of being handled and/or vaccinated and the immediate APR response following these procedures.

The dual vaccine chosen for this study is routinely used in chicken production. Vaccination routes recommended for this vaccine are by spray, in drinking water or by eye drop. Intraocular vaccination (eye drop) was selected in this study to ensure every individual bird received the same dose, which could not be guaranteed with the other routes. To evaluate the success of the vaccine, the amount of specific antibody (IgY) raised against the immunogen proteins of the vaccine after 21 d, was also determined.

3.2 Materials and Methods

3.2.1 SPF Chicks and Housing

One hundred and eighty 1-day old SPF White leghorn layer chicks were hatched out at the experimental farm (Cochno Research Farm, University of Glasgow) and divided into two batches each containing 90 chicks. Each batch of chicks was then placed in a separate controlled environmental room (R1 an R2) in one of two pens (n=45 per pen). The two pens in each room had a litter of wood shavings and were fitted with a brooding ring. The stocking density was 12chicks/m^2.

The chicks were fed *ad libitum* with a commercially available chick crumb formulated to meet or exceed National Research Council (NRC, 1994) guidelines and the birds had access to fresh water throughout the study period. The light, temperature and ventilation within each room were automatically controlled and adjusted according to management guide recommendations. The chicks were allowed to adjust to their environment for the first 7 days before the experiment commenced. Strict biosecurity was applied to prevent cross contamination between the two rooms and each pen, thus the controls (R2) were always visited first and then the vaccinated room (R1).

3.2.2 Experimental Design

The experiment commenced when the chicks were 7 days old. There were 9 sampling time points; pre (0) and post vaccination (PV) at 12 h, 24 h, and then 2, 3, 4, 5, 6, and 21 days thereafter. At each sampling time point 12 chicks were weighed and culled and samples collected, 6 per treatment and 3 per replicate pen. Full ethics approval was granted in advance by the School of Veterinary Medicine Ethics Committee, University of Glasgow.

3.2.3 Vaccine and Vaccination

A commercially available combined Newcastle disease and Infectious Bronchitis (N/B) Live, freeze-dried virus vaccine (Nobalis Ma5+Clone 30, MSD Animal Health) was used in this experiment. After re-constituting the vaccine in sterile saline solution, each dose contained at least $10^{3.5}$ EID_{50} of the IB strain Ma5 and 10^6 EID_{50} Newcastle disease virus strain Clone 30.

The chicks in pens 1 and 2 in R1 were weighed and the vaccine was administered by the intra-ocular route with one drop applied to one eye. A sterile saline solution was administered by the same route to control animals in pens 1 and 2 housed in R2.

3.2.4 Blood Sampling and Assessment

At each sampling time point three chicks were chosen from each replicate pen, weighed then humanely culled by dislocation of the neck followed by decapitation. Following decapitation, approximately 1.5 ml of blood was collected from the major vessels in the neck using heparinized tubes. Fresh blood was used to make blood smears to determine the H/L ratio; the remainder was centrifuged (3000 × g) for 15 min at 4^{o}C and the plasma immediately frozen at -20 ^{o}C. SAA, AGP, and CP levels were measured in all samples collected using commercially available ELISA kits as described in section (2.2.1 and 2.2.2). OVT was measured using a radial immunodiffusion assay as described below. Plasma from samples on day 0 and 21 days were used to estimate the antibody titres as detailed below. 1D gel electrophoresis was also used to assess plasma protein bands from both groups obtained on selected days (1,2 and 6) for running 1D gel see section 2.2.5).

3.2.5 Heterophil / Lymphocyte Ratios

Differential WBC counts were carried out on the blood smears stained with the May-Grunwald-Giemsa stain. Two hundred leucocytes were counted and classified into lymphocyts, heteropils, monocyts, eosinophils and basophiles and count how many of each per slide by using microscobe with 100x lense. The H/L ratio was calculated by dividing the total number of Heterophils by the total number of Lymphocytes (Gross and Siegel, 1983; Ohara et al., 2015). Samples used for this technique were at 0, 1, 2, 3, 4, 5, 6-day post vaccination but due to technical issues it was not possible to use the 12 h post vaccination samples. A representative example of the histology slide is shown in Figure 3.1.

3.2.6 ELISA for SAA, AGP and CP

The Enzyme Linked Immunosorbent Assays ELISA assays for chicken APP (AGP, SAA and CP) were obtained from Life Diagnostics Inc. They were performed according to the manufacturer's instructions with a dilution factor for the plasma samples of

1:10000 for AGP, 1:40000 for CP (section 2.2.1), and 1:20 for SAA (section 2.2.2). In brief, two samples from each time point from both vaccinated and unvaccinated birds were run in one plate and tested against the provided standard, so in total, one plate of ELISA is used for running to test 37 samples in addition to two samples for high and low quality control obtained from previous study. Since samples from the same time point were assayed on different plates, the internal quality control samples were compared between plates to ensure precision of the analysis. Each individual sample and the standard were diluted by the dilution buffer and were run in duplicate. For the SAA ELISA, 50µl of plasma samples were heated (instead of 100µl as instructions says) before dilution and running the assay, and the rest of the sample was discarded.

3.2.7 Radial immunodiffusion (RID) assay for OVT

A 1% of agarose gel was prepared by dissolving 0.2 g of agarose (sigma A9539-logRK0015) in 20ml of TBS (appendix B section 2.8) then placed in a conical tube and brought to temperature in a water bath at 56^{o}C. 0.2 ml of Sheep α-chicken ovotransferrin antibody, previously described by O'Reilly (2016) was then added and mixed gently. The mixture was poured onto a Sigma Aldrich electrophoresis film for agarose gels (Batch number: 110EO264-100EA) and allowed to cool down to room temperature before transferring to a cold room (4^{o}C) for 10-15 minutes. Four wells (with diameter 2.8 mm) one for each standard curve and three wells for each sample were then punched into the gel. 5ul of each standard or sample was subsequently loaded into each well. The concentrations of standard used were: S1=2.5mg, S2=1.25mg S3=0.65mg and S4=0.32mg. The gel membrane was kept in a humid chamber overnight at room temperature. The gel was washed in 0.09% NaCl overnight, and the following day used filter paper and tissue to dry with pressure, to remove all the liquid from the agarose. The gel was washed in 0.09% NaCl for an h and pressure dried to remove liquid from the gel. This was repeated once further. The fourth and final washing of the gel was by dH_2O for 1 h, pressure dried and left on bench to air dry overnight. Next 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 h. The gel was left to dry overnight. To determine the OVT concentration of samples against the

standard was generated by measuring the ring diameters and plotting diameter against the concentration.

3.2.8 One D Gel Electrophoresis SDS-PAGE

Additional changes in blood plasma proteins arising from the vaccination procedure were examined by 1D gel electrophoresis SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) as described in section (2. 2. 5). Pooled samples from both vaccinated and control birds from days 1, 2 and 6 were used based on the outcome of ELISA assays.

3.2.9 Antibody titre raised against the vaccine

A modified antibody (Ab) titration assay (Crowther, 2009; Snyder et al., 1983) was used to determine the amount of IgY Ab raised against the vaccine in this study. A 96-well plate (Costar Assay Plate, CORNING) was coated with the Newcastle disease and Infectious Bronchitis vaccine (Nobalis Ma5+Clone 30) diluted to a protein concentration of 20µg/ml in 0.2M bicarbonate buffer at pH 9.5 (appendix B section 2.5) and incubated at 4^{o}C overnight. The protein content of the vaccine had been determined by a Bradford protein assay (Sigma-Adrich, Dorset, UK), with bovine serum albumin standard according to the manufacturer's instructions. Each well was then aspirated and washed 4 times using Tris-buffer saline (TBS) 50 mM Tris-Cl, pH 7.5 containing 0.05% tween-20. Wells were blocked with 200µl of 5% (w/v) Marvel milk protein diluted in TTBS (0.05%) (appendix B section 2.3) for 1h at room temperature on a rocking plate. The plate was then washed as above.

Antibody standards were made by serially diluting pooled samples collected from vaccinated birds sampled at 21days post vaccination and by diluting 1:20 in TTBS with 0.5% Marvel milk. This was used as the standard 1 (S1) and given a value of 100 arbitrary units (AU) of antibody. It was then diluted in a 6-fold serial dilution to S6 (3.13 AU) using TTBS with 0.5% Marvel milk. For the analysis all chicken plasma samples were diluted 1:80 in TTBS 0.5% Marvel milk. To duplicate wells a 100 µl aliquot of diluted standard or sample was added. After 1h incubation with constant shaking at room temperature, the plate was washed as above. The second antibody, anti-Chicken IgY VHH Single Domain Antibody conjugated to horse radish peroxidase (HRP) (Abcam, Cambridge, UK), was diluted to a concentration of 1:5000 with TTBS

0.5% Marvel milk and added before incubating for another 1 h at room temperature by constant shaking then washed as above. 100µl of tetra-methyl benzidine (TMB, KPL laboratories, Inc., Maryland, USA) was then added to each well for 20 minutes at room temperature whilst rocking until a blue colour developed, then 100µl of stop solution (2M H_2SO_4) was added (appendix B section 2.7). This caused the colour to change to yellow. The absorbance of the resulting solution was measured at 450nm using an OPTIMA absorbance microplate reader (BMG Labtech Ltd, Bucks, UK). A standard curve using 4-parameter fit curve optima software was used to determine the antibody response. Specificity of the antibody was confirmed by western blot (for method see chapter 2 section 2.2.1.). Two gels were prepared (Gels A and B), these were coated with three different dilutions of the N/B vaccine (1:2, 1:4, and 1:8). The chicken plasma from day 21 were then added to gel A at 1:80 dilution, while gel B was loaded with TTBS as a negative control. Anti-chicken IgY-HRP secondary antibody was added to both gels and developed.

3.2.10 Data handling and statistical analysis

Results obtained were initially stored in Excel (Microsoft 2010) and simple descriptive statistics were obtained. Data were later exported to minitab statistical package software; version 17.1.0 for further analysis. Tests for normality were run on the acute phase proteins (SAA, AGP, CP, and OVT) H/L ratios and the antibody values using normality test and quantile-quantile (Q-Q) plots. To analyse effects on blood APP, H/L ratio and antibody titer of vaccine treatment, For variables which were not normally distributed, non-parametric tests (Mann-Whitney's test for two groups) were used to evaluate the differences between groups (vaccinated and control birds). The weight of the birds in each group that were culled at each time point were compared by ANOVA. P-value was considered significant at <0.05.

3.3 Results

3.3.1 Chick Weights

The chicks in each group were within the same weight range when the experiment commenced at day 7 (Table 3.1) and there were no significant differences in weight observed between the groups in response to vaccination. Over all, the increase in weight in each group was as expected.

Table 3.1 Body weight.
Median and range of values are for n=6 control and vaccinated birds culled at each sampling time point.

Age/ day	Sampling time (day)	Control Median (range)(n=6)	Vaccinated Median (range) (n=6)	P value
7	0	69.85 (56.5-84.2)	76.4 (60.8-81.2)	0.5752
8	1	77.55 (68.2-87.6)	78.6 (66.6-99.1)	1.0000
9	2	85.1 (77.8-97.1)	85.2 (79.4-94.3)	0.7488
10	3	92.7 (60.5-102.5)	95.45 (80.7-100)	0.0927
11	4	105.1 (90.4-122.3)	96.3 (58.9-109.6)	0.0929
12	5	111.35 (93.2-124)	103.5 (90.8-132.1)	0.4712
13	6	125.05 (95.6-138.9)	122.7(91.1-142.7)	0.2980
28	21	332.95 (294-390.7)	297.1 (271.5-387.9)	0.0957

3.3.2 H/L Ratio

In the vaccinated birds there was a significant increase in the number of Heterophils and a significant decrease in the number of lymphocytes on days 1 and 2 post vaccination (Table 3.2). White blood cells pictures from stained slides shown in Figure 3.1. Likewise, the H/L ratio increased significantly ($P<0.01$) to reach a peak of 0.58 (0.39-0.65) median (range) in the vaccinated group (V) compared to the control group (C) 0.20 (0.08-0.32) median (range) by 1 day post treatment (Table3.2). This increase was also significant on day 2 ($P<0.01$) and day 3 ($P<0.05$) post treatment. From day 4 post vaccination there was no significant difference in the H/L ratio between the control and vaccinated groups examples of chickens white blood cells are presented in Figure 3.1.

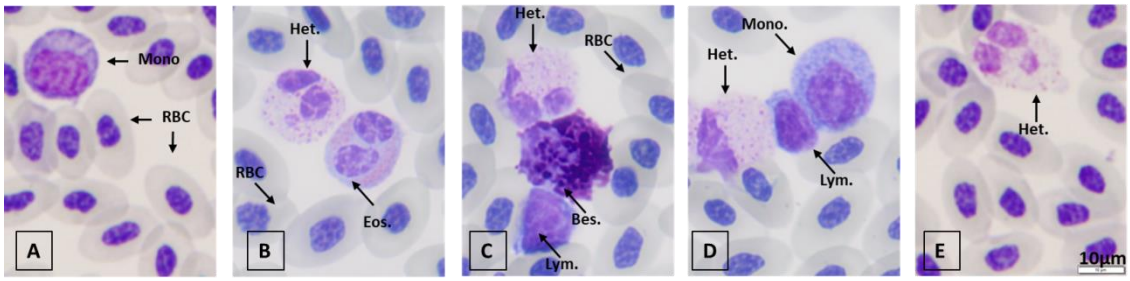

Figure 3.1 Chickens white blood cells.
Slides stained with May-G stain, power of magnification is 100x. shows an examples of different white blood cells of chickens, picture A (Mono.= monocytes, RBC= red blood cells), picture B (Het.= heterophils and Eos.=eosinophils), picture C (Bas.=basophile, Lym.= lympholcytes and Het.= heterophils) and picture E (Het.= heterophils) .

Table 3.2 Heterophil (H) and lymphocyte (L) counts in response to the vaccine
The table shows median (range) of H, L with statistical differences (P value) for each time point. Statistical differences were marked as *=P<0.05 and **= P<0.01between groups

Day post treatment	Control H. Median(range) X10⁹ /L	Vaccinated H. Median(range) X10⁹ /L	P value	Control L. Median(range) X10⁹ /L	Vaccinated L. Median(range) X10⁹ /L	P value
0	7.4 (6-9)	9.3 (6-12)	0.1441	86.4 (84-89)	83.7 (82-87)	0.0679
1	14.8 (11-22)	28.6 (23-32)	0.008**	75.1(64-86)	52.8 (49-52)	0.008**
2	16.0 (13-20)	25.7 (21-36)	0.010**	77.8 (73-82)	57.7 (52-62)	0.008**
3	8.8 (9-14)	20.0 (14-33)	0.054	85.0 (73-97)	68.7 (51-94)	0.0656
4	13.2 (11-14)	10.2 (4-19)	0.1282	81.8 (79-83)	80.8 (70-90)	0.5752
5	14.3 (13-22)	7.7 (5-12)	0.0656	79.5 (70-96)	83.3 (77-87)	0.1495
6	13.5 (7-22)	7.0 (4-11)	0.0547	81.3 (73-90)	86.2 (82-93)	0.1495
21	4.2 (2-7)	5.2 (3-11)	0.9273	93.5 (89-97)	87.6 (83-92)	0.0828

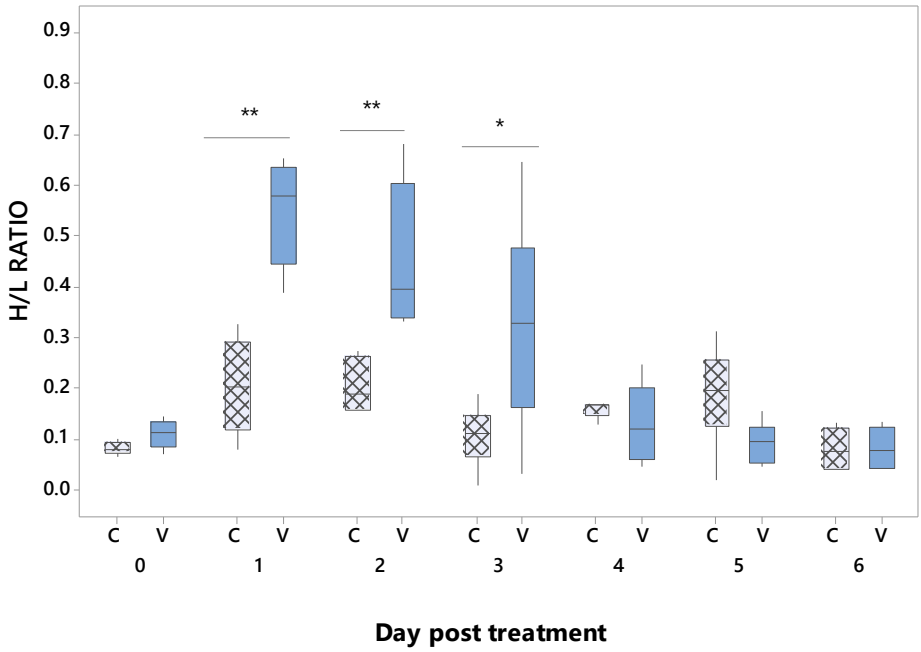

Figure 3.2 The Heterophile/Lymphocyte ratio.
H/L ratio was significantly higher in the vaccinated group (V) (n=6 per time point) at 1, 2 and 3 days post treatment compared to the control group (C) (n=6 per time point). (Horizontal bars indicate statistical differences between groups (** = P≤0.01, * = P≤0.05). Data at each sampling time point are presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

3.3.3 Acute Phase Proteins

Alpha-1 Acid Glycoprotein (AGP) in the vaccinated group was statistically higher than the AGP levels in the control group on day-2 post treatment. The AGP concentration (median) increased 2-fold, from 0.47 g/L to 1.007 g/L, and this increment is statistically significant, P<0.05 (Figure 3.3) At day-3 post treatment the AGP levels were not statistically different from the control animals. At day-6 post treatment, another significant increase (P< 0.05) in the levels of AGP in the vaccinated group over controls was observed with a 1.9-fold increase from 0.54g/L (0.37-0.68) median (range) in the control group to 0.99g/L (0.53-1.29) median (range) in the vaccinated group.

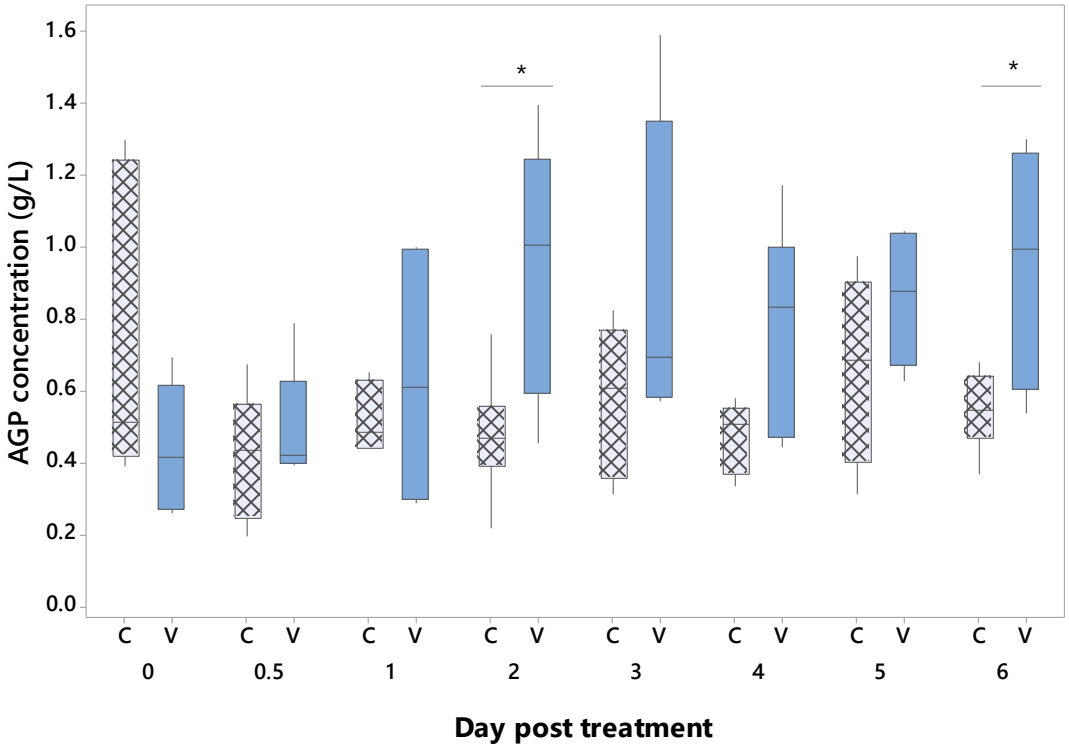

Figure 3.3: AGP response to the N/B vaccine.
Comparison of AGP concentrations in vaccinated (V) (n=6 per time point) and control groups (C) (n=6 per time point) over the time course of this study. Significant differences (Horizontal bars) were detected at day 2 and day 6 post treatment (* = P< 0.05). Data at each sampling time points are presented in median with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

Serum Amyloid-A (SAA) The SAA levels were significantly higher in the vaccinated group on day-1 post treatment (P< 0.05) with a 2.5-fold, increase from 39.5 µg/L (20.6-63.7) median (range), in the control group to 103.5 µg/L (61-180.9) median (range), in the vaccinated group. Thereafter there was no significant difference except on day-6 post vaccination when the SAA concentration was significantly higher in the vaccination group with a 2.8-fold (P<0.01) increase from 42.5 µg/L (22.7-63.3) median (range) in the control group to 105.5 µg/L (63.8-238.1) median (range) in the vaccinated group (Figure 3.4).

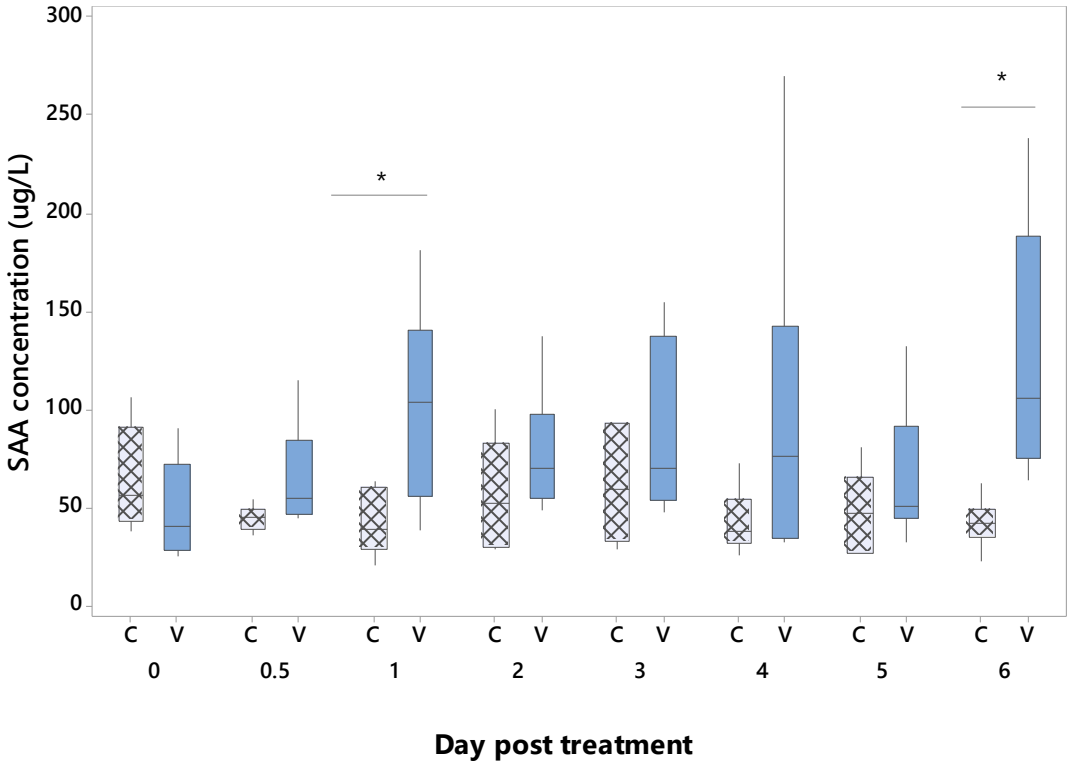

Figure 3.4 SAA response to the vaccine.
Comparison of SAA concentration in control (C) (n=6 per time point) and vaccinated groups (V) (n=6 per time point) over the time course of this study. Significant differences were detected (Horizontal bars) at day 1 and day 6 post treatment, ** = P≤0.01, * = P≤0.05. Data at each sampling time points are presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

The plasma levels of CP and OVT were not statistically different in the vaccinated group compared to the control group at any time points over the course of the study (Figure 3.5 and Figure 3.6).

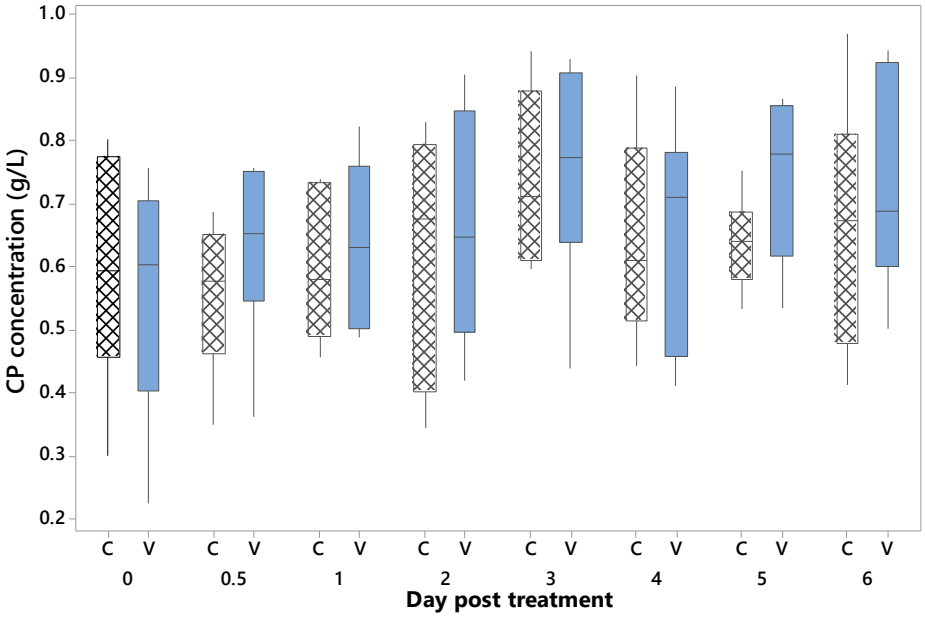

Figure 3.5 Ceruloplasmin levels in response to the N/B vaccination.
Comparison of CP concentration in control (C) (n=6 per time point) and vaccinated groups (V) (n=6 per time point) over the time course of this study. No significant differences were detected at any time points. Data of 8 sampling time points are presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

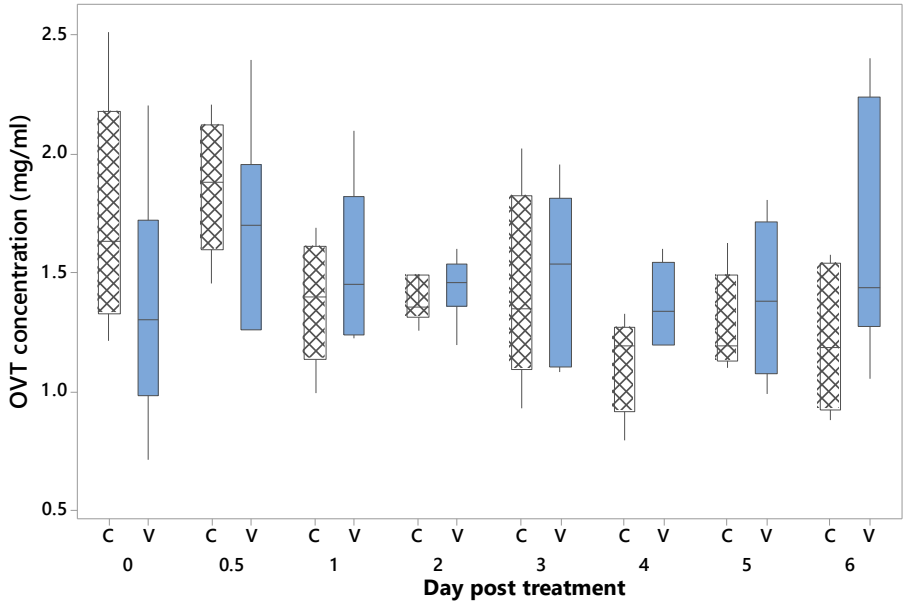

Figure 3.6 OVT response to the N/B vaccination.
Comparison of OVT concentration in control (C) (n=6 per time point) and vaccinated groups (V) (n=6 per time point) over the time course of this study. There no significant differences were detected at any time points. Data at each sampling time point are presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles.

3.3.4 SDS PAGE Electrophoresis

The results of SDS-PAGE for pooled plasma samples collected on days 1,2 and 6 in the control and vaccinated groups are presented in Figuer 3.7. No obvious differences were observed in the most abundant proteins were detected.

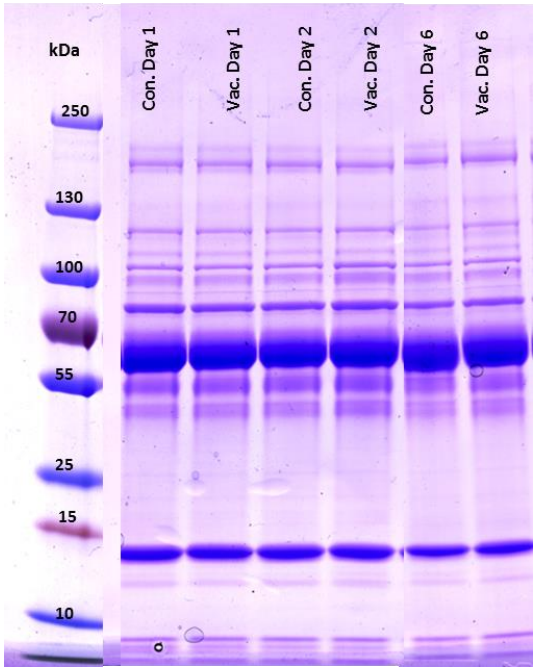

Figure 3.7 Electrophoresis of sera sampled from vaccinated and control birds.
SDS-PAGE of pooled samples of control (Con.) and vaccinated (Vac.) samples at day 1, 2, and 6 post treatment. Shows bands of the proteins separated according the molecular weight.

3.3.5 Antibody Titre Raised Against the Vaccine

The western blots shown in Figure 3.8 confirm the specificity of the antibody raised in the vaccinated birds. Bands only developed in blot A which the chicken plasma from vaccinated birds (n=6) at 21 days were added. No bands were formed in blot B where the primary antibody (plasma) had been omitted (Figure 3.7 B). The antibody titre was significantly elevated by 3.1-fold ($P<0.01$) after 21 days from a median and range of 154 AU (130-186) to 488AU (346-781) in the vaccinated group (Figure 3.9).

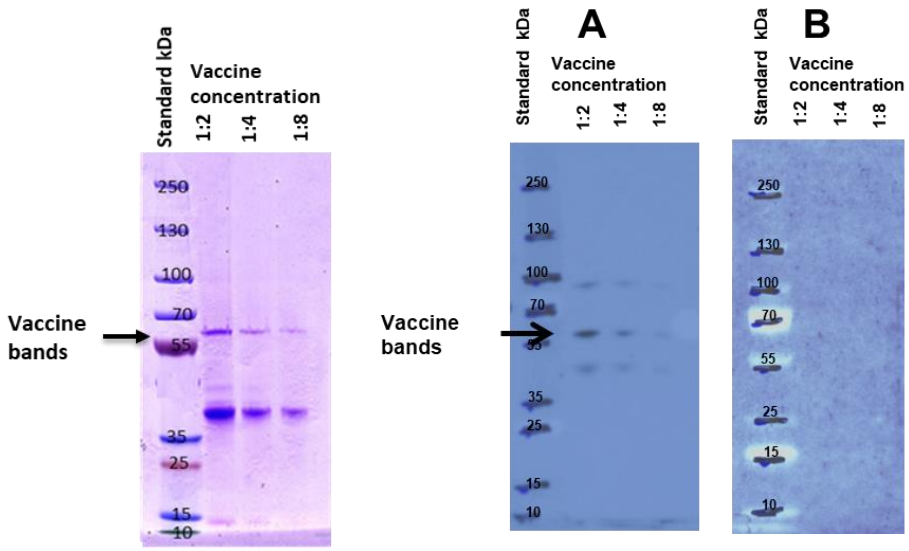

Figure 3.8 SDS-PAGE and western blot of the antibody specificity to N/B vaccine.
The left figure is the SDS-PAGE gel of the diluted vaccine in different dilution factors, which shows the vaccine bands before transferring them into the blot paper. The right figure is the blots A and B were coated with three different dilutions of the N/B vaccine (1:2, 1:4, and 1:8). The pooled chicken plasma from vaccinated birds at day 21 (n=6) was loaded to blot A at a dilution of 1:20 dilution, while blot B was loaded with TBS-T as a negative control. Anti-chicken IgY-HRP secondary antibody was added to both blots.

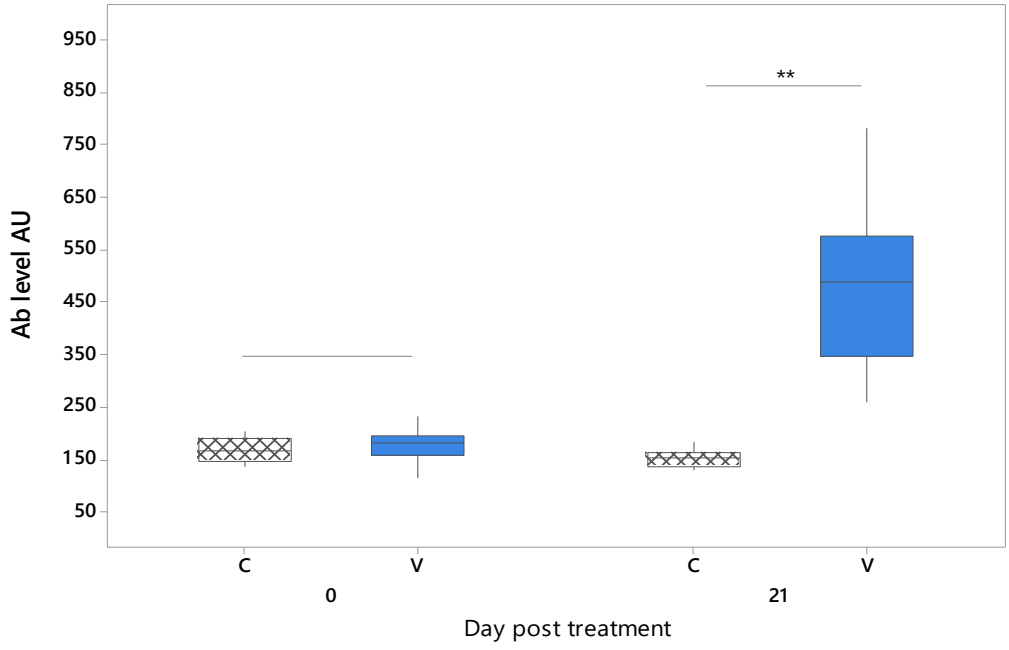

Figure 3.9 Antibody levels raised against the N/B vaccine.
The antibody levels in pooled plasma from n=6 vaccinated and control birds at day 0 (pre-treatment) and day 21 post treatment. Horizontal bars with star indicate statistical differences between groups (** = P≤0.01). Data at each sampling point is presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles. T-test Man-Whitny test to the difference between two groups.

3.4 Discussion

This investigation showed that an intra-ocular vaccination of SPF layer chicks with a dual vaccine against Newcastle disease virus and infectious bronchitis (N/B) causes an immediate but mild APP response, with small increases in AGP and SAA on day 2 and day 1 post vaccination respectively (section 3.3.3). The vaccinated birds also underwent a mild vaccination stress response with H/L ratios remaining higher in the vaccinated birds for up to 3'd day post treatment (Figure 3.2). The efficacy of the vaccine to produce antibody to the immunogens contained, was confirmed by demonstration of the presence of specific antibody on day 21 post vaccination (Figure 3.9).

The SAA concentration in many species is related to the severity or virulence of the pathogen, and it can be used as marker for detection of inflammation (Eckersall, 1995; Ceron et al., 2005), serving as an indicator of the immediate innate immune response to stimulations such as vaccination. SAA usually increases within several h (5 to 6 h in humans) of an inflammatory event and decreases after 48 h and typically increases 10- to 100-fold during a response (Kushner and Rzewnicki, 1994; Gruys et al., 2005). In the current study, the intraocular administration of N/B vaccine stimulated only a mild increase in the SAA concentration, though the plasma SAA increased significantly at 24 h post treatment, with a 2.4-fold elevation, as is seen with vaccination in other species. For instance, SAA levels increased sharply from 5 h to hit the peak at 24 h to be 3.6-fold higher than the previous level before inoculation following intratracheal inoculation of beef calves with inactivated *Pasteurella multocida* (Dowling et al., 2004). In lambs, subcutaneous vaccination with Heptavac P® (combined *Clostridia* and *Pasteurella* vaccine) also caused a significant increase ($P < 0.01$) in both SAA and haptoglobin (Hp). The level of SAA peaked at 24 h, reaching a 400-fold increase, and did not return to normal concentration until 4 days later (Eckersall et al., 2008). In the current study, a second elevation of SAA in the vaccinated group compared to the control group occupied on day 6 post treatment, which also coincided with a second peak in AGP concentration. A limitation of this study was that it was not possible to repeat sample the same individual chicks at each of the specified time points, so it is not possible to determine if this second peak in APP was present because some individuals responded more slowly to the vaccination or if some other factor was

coming into play, e.g., social stress. The most likely explanation is that the second APP peak was due to individual bird differences at same individual could be hyper or hypo responders in the APP (Verschuur et al., 2004; Elsasser et al., 2005).

In the current study, AGP in the vaccinated group was significantly higher by a 2-fold increase at 2 days post treatment. This result is similar to that reported by Sylte and Suarez (2012), who reported an AGP peak serum concentration of 2.6 -fold at 48 h post experimental infection with influenza virus in 4 wks old White Leghorn chickens. However, a prolonged increase in AGP unlike the (96 h) change as reported by these authors was not observe in the current study. Notably the intraocular inoculation with a vaccine strain of Gumboro disease virus lead to a 2.4-fold increase in plasma AGP at day 4 post treatment in 3 wks old White Leghorn SPF chickens (Inoue et al., 1997) whereas intraocular inoculation with a highly virulent strain of Gumboro disease virus lead to an increase of plasma AGP at day 2 which peaked at day 6 post treatment (6.2-fold increase).

OVT and CP concentration remained at low levels throughout this study (Figure 3.5 and Figure 3.6). No additional protein bands were identified in pooled samples of plasma analysed by 1D gel electrophoresis at 1, 2 and 6 days post vaccination. Where the results of 1D gel were shown no differences in bands of vaccinated bird samples compared to control. These results support the conclusion that there was only a mild APR.

Measuring the H/L ratio has been established as means of evaluating stress in chickens (McFarlane and Curtis, 1989; Maxwell et al., 1992; Puvadolpirod and Thaxton, 2000; Post et al., 2003). In the current study, an increase in heterophils and a decrease in lymphocytes was observed in the vaccinated group, whereas the control animals did not show any significant change in their H/L ratios. The changes observed in the H/L ratios were therefore likely to be related to the vaccine action and its effects on the innate immune response. These inflammatory mediators initiate and modulate the APR, which by diffusing into the extracellular fluid and circulating in the blood, leads to the activation of the hypothalamic-pituitary-adrenal axis, decrease production of growth hormone and physiological changes as fever, lack of apatite and catabolism of muscle cells (Gruys et al., 2005). As a result, inflammation may have a combined effect on the H/L ratio by increasing heterophil

concentrations due to increased granulocytopoiesis and decreasing lymphocyte concentration due to cytokine-mediated increases in corticosterone one concentration (Clark, 2015). Change of the H/L ratio has been reported in birds to be 0.19 to 64.67 in different diseases (Clark, 2015), so for our vaccinated birds, a change in H/L from 0.11 to 0.54 is suggestive of a mild stress response.

In conclusion, vaccination of chicks with N/B vaccine by the intra-ocular route has produced a mild acute phase response and vaccination stress response in 7-day old SPF layer chicks. Of the two methods, the H/L ratio was more sensitive and consistent in terms of measuring the mild stress response induced by the vaccination procedure under the conditions employed in this experiment. For future work, it will be of interest to monitor the APR in the same individuals using different vaccines and routes of administration and to assess whether the post vaccine APP response is correlated to both the H/L ratio and the subsequent antibody titre.

Chapter 4 Serum and acute phase protein changes in laying hens, infested with poultry red mite.

4.1 Introduction

This chapter describes a time course study in which 3 APP and the serum proteome of commercial layers are characterised before and after a PRM challenge at 18.5 wks of age. Poultry red mite (PRM) (*Dermanyssus gallinae*) is one of the most important ectoparasites affecting the global poultry industry and is a haematophagous ectoparasite of both domestic poultry and wild birds (Brannstrom et al. 2008; Kristofik et al., 1996). In Europe the prevalence of PRM in commercial laying facilities is estimated to range from 4 to 100% in several European countries (Sparagano et al., 2014) and is present in all production types. PRM reside most of the time off-host in the structure and furniture of poultry housing and emerge only in darkness to feed on the hens every 2-4 days (Maurer et al., 1988). This makes their detection and control problematic. Conditions within poultry houses are well suited to PRM population growth, where temperatures between 10^{o}C and 35^{o}C, and high relative humidity (>70%) facilitate PRM reproduction and development (Maurer and Baumgartner, 1992; Nordenfors et al., 1999). Indeed, weekly doubling of populations has been reported in some egg-laying facilities (Maurer and Baumgartner, 1992). PRM densities commonly reach up to 50,000 mites per bird in caged systems, and densities can reach 500,000 mites per bird in severe cases (Kilpinen, 2005). PRM are present all year-round, but the highest densities occur during hot and humid seasons (Othman et al., 2012).

4.1.1 PRM lifecycle

The lifecycle of the PRM is described in Figure 4.1. Complete development of PRM, from egg to adult through one larval stage and two nymphal stages, typically occurs over two weeks (Axtell, 1999). *Dermanyssus gallinae* is an ectoparasite (lives or feeds on exterior of the host) that typically feeds at night (Sparagano et al. 2014). It does not stay on the bird at all times, and rarely feeds during the day. The adult measures about one millimetre long (Knezevic et al., 2017). After feeding, adults are red, but look black, grey or white without host blood in their system (Hoy, 2011). PRM lay eggs where they hide, in areas such as cracks, crevices, and litter. Females lay eggs in clutches of four to eight, generally laying around 30 eggs in their lifetime (Chauve, 1998). After hatching, the six-legged larvae are sluggish, and molt after one day. The eight-legged protonymph feeds and molts to an eight-legged deutonymph, which then feeds and molts to an adult. Removing the host from an

area will not eliminate the mites. The deutonymph and adult are known to resist desiccation and live as long as eight months without feeding (Chauve, 1998). The introduction of *D. gallinae* into poultry houses could be via the trade route (i.e., the movement of birds, egg crates, etc., between premises) (Oines and Brannstrom, 2011), or infestation via both trade and wild birds which was reported in Brazil (Roy and Buronfosse, 2011). PRM is primarily considered a pest of chickens. Nevertheless, it feeds on at least 30 species of birds including pigeons, sparrows, rock doves, and starlings (Sparagano et al., 2014, Proctor and Owens, 2000). It has also been known to feed on horses, rodents, and humans (Sparagano et al., 2014)

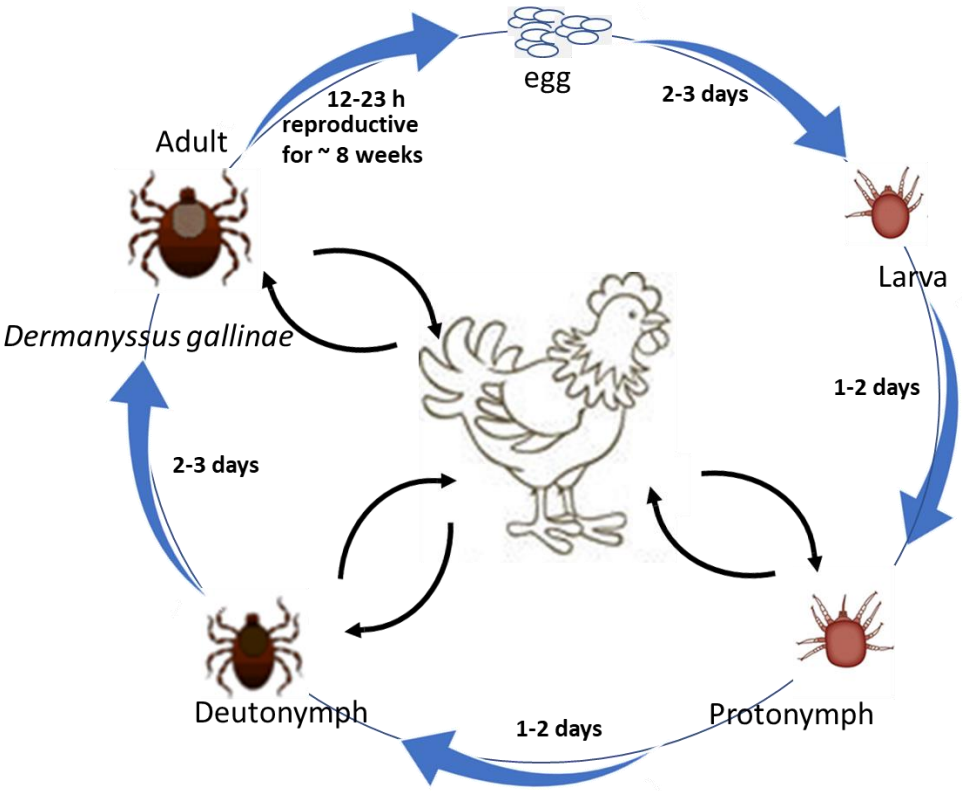

Figure 4.1 Life cycle of the poultry red mite.
Dermanyssus gallinae, under favourable conditions (adapted from Sparagano et al. 2014). Schematic of the PRM life cycle: Eggs of PRM are laid in clutches (4–8 eggs) in refugia where larvae may remain without feeding prior to their first molt. The protonymph, deutonymph and adult stages all feed on the host chicken. Each female may lay up to eight clutches of eggs between feeding bouts, typically laying around 30 eggs in a lifetime.

4.1.2 Control of PRM

Traditionally, PRM control relies upon synthetic acaricide spraying of poultry houses and equipment; however, resistance against these compounds has resulted in efficacy reduction (Beugnet et al., 1997; Sparagano et al., 2014). This together with concerns over human safety and environmental contamination means that the number of acaricides licenced for PRM treatment is now restricted in the European Union (EU) (Flochlay et al., 2017). Nevertheless, evidence of illegal use of banned acaricides including detection of pesticide residues in the organs and tissues of laying flocks in several EU countries is evident (Marangi et al., 2012). Fipronil residues for example have recently reported in eggs from laying flocks in several EU countries (Anonymous, 2017). The development of novel acaricides which make use of biopesticides or plant-based products, or biological agents such as entomopathogenic fungi, nematodes and bacterial endosymbionts offer alternative approaches to the control of PRM but many of these are still at the pre-commercial stage and therefore not widely available (Sparagano et al., 2014). Vaccination as a control strategy offers several advantages over existing treatments, but vaccine development against arthropods is notoriously difficult (McDevitt et al., 2006; Willadsen, 2004) and for PRM it is further hindered by the relatively poor understanding of the mite-host relationship (Harrington et al., 2009; Bartley et al., 2017).

4.1.3 PRM, bird health and welfare

PRM cause a significant deleterious effect on their avian host, such as a high level of psychogenic stress (Kowalski and Sokol, 2009), a decrease in egg production, anaemia, blood staining of eggs, and an increase in mortality rate (Chauve, 1998; Kilpinen, 2005). PRM is a known vector (transmitter) such as fowl pox virus, Newcastle virus, and fowl cholera (Hoy, 2011). It has been reported that PRM could human an attached on, as an occupational hazard for poultry workers (Cafiero et al., 2011). Outside the poultry sector, attacks have been reported in private residences, hospitals, and office spaces due to infested birds (Bellanger et al., 2008; Cafiero et al., 2011; Lucky et al., 2001; Rosen et al., 2002). Effective control of PRM is therefore potentially important not only in the poultry sector, but also in other sectors, human health included.

4.1.4 PRM and public health

D. gallinae is involved in the transmission of numerous poultry pathogens, including zoonotic pathogens like *Salmonella enteritidis*. De

Dermatitis caused by those mites elicited very similar skin lesions and lasted from a few days up to several weeks. Similar findings in cases of dermatitis caused by *Ornithonyssus bacoti* have also been reported (Mumcuoglu et al., 1983). They are also capable of biting humans, Dermatitis caused by those mites elicited skin lesions and lasted from a few days up to several weeks, originated from various hosts such as birds and rodents (Rosen et al., 2002). In most cases the lesions disappeared without any specific therapy after a few days of continuously avoiding contact with mites (Rosen et al., 2002). Whereas, Kozdrun et al. (2015) have reported that reactions occurring in human skin are not specific and difficult to diagnose. They are manifested by pruritic papules and occasionally by vesicles, urticaria, and or erythema. The mites are most commonly seen on the legs, but not on the skin between the fingers or genital skin. Poultry farmers are also exposed to infection with the mites (Haag-Wackernagel, 2005).

4.1.5 PRM and acute phase protein response

The use of acute phase proteins as biomarkers for assessment of overall health and welfare has potential for diagnosis and prognosis in veterinary medicine (O'Reilly and Eckersall, 2014; Ceciliani et al., 2012; Ceron et al., 2005; Cray et al., 2009), thus allowing the more rational and targeted use of drugs and treatments. Kowalski and Sokol, (2009) have hypothesised that the hens infested with PRM would have higher concentration of circulating APP, and that PRM infestation may be associated with proinflammatory cytokines. In addition to APP, there has been growing interest in the use of proteomic analysis to assess changes in the serum proteome (Eckersall and McLaughlin, 2011), of poultry and other livestock in order to identify additional biomarkers of disease (Almeida et al., 2015). In commercial layers the onset of lay requires a major change in the physiology of the hen, with nutrients such as lipid and protein being required in copious amounts for egg production. With the exception of immunoglobins, synthesis of most of the major egg yolk proteins (Vitellogenins and very low-density lipoproteins VLDLs) takes place in the liver predominantly under the regulation of estrogen and to a lesser extent androgen.

These egg yolk precursors are then transported in the blood to the ovary where they are further processed to phosvitin, lipovitellin, triglycerides, cholesterol, and phospholipids within the developing oocyte (Bourin et al., 2012). To date there have been few investigations of the alteration this causes in the serum proteins of hens as they approach sexual maturity. Liou et al. (2007) however did report that Vitellogenin and Apolipoprotein-A1 changed dramatically at peak egg production relative to initial egg production and went on to demonstrate an association between these proteins and egg production in different groups of Taiwan red-feathered country chickens. In the current study we wanted to evaluate if a PRM challenge disrupts the transport of these and other major proteins in the blood serum required for egg production.

The aims of this study were therefore i) to investigate the APP response over the time course of an experimental PRM infestation in laying hens ii) to demonstrate the major changes in serum proteins following the onset of lay and iii) to evaluate if a PRM infestation significantly alters the serum protein profile of laying hens.

4.2 Materials and methods

4.2.1 Treatment and sampling

This study was performed using serum samples and additional data collected from control hens which were part of a PRM vaccine field trial carried out by the Moredun Research Institute (Bartley et al., 2017). The hens were Lohmann Brown Hens (n=384) which were "paired" with cages of fully vaccinated birds, the latter were not part of this study. The 'controls' in this vaccine field trial, received two thigh injections of a 0.5 ml dose of a vaccine placebo of Montanide ISA70VG adjuvant (SEPPIC, Terrassa Bellini - Paris) formulated in 7:3 ratio with 10 mM Tris–HCl; 0.5 M NaCl, pH 7.4 at age 12 and 17 weeks of age. At the time of the 2nd placebo vaccine injection (week 17) the birds were placed in cages with four birds per cage in a particular spatial arrangement (Bartley et al., 2017). At 18.5 weeks of age, all 96 cages were subsequently infested with PRM (10,000 live mites per cage). This infestation rate was used so that the experimental cages were not immediately overwhelmed with mites (Bartley et al., 2017). Every two weeks for four and a half months post challenge (Figure 4.2) the PRM populations were estimated using a trapping and counting procedure (Bartley et al., 2017). Plastic ADAS Mite Monitor traps (ADAS Ltd., Oxon, UK) were fixed to the egg collection tray at the front of each cage. After 24 h the mites in the traps were collected into individually-marked plastic containers containing 70% (v/v) ethanol and the mites were counted.

Blood samples were collected at a) placebo vaccination 1 (pre-PRM challenge, week 12); b) placebo vaccination 2 (pre-PRM challenge, week 17); c) 5 days post -PRM challenge (week 19); d) at times which coincided with alternate PRM trap evaluations viz. 23, 27, 31, 36 and 38 weeks post PRM challenge (Figure 4.2). At each sampling time point, blood was collected from 8 randomly selected birds by bleeding directly from the wing vein into a non-heparinised tube. The blood (1ml) was then allowed to clot at 4^{o}C for 24 h and the sera were obtained following centrifugation at 3000 g for 10 minutes. Sera were stored at -20^{o}C, until required for further analysis.

The PRM experiment performed under the terms of UK Home Office licence (PPL 60/ 4324) and the experimental design was ratified by the ethics committee of the Moredun Research Institute.

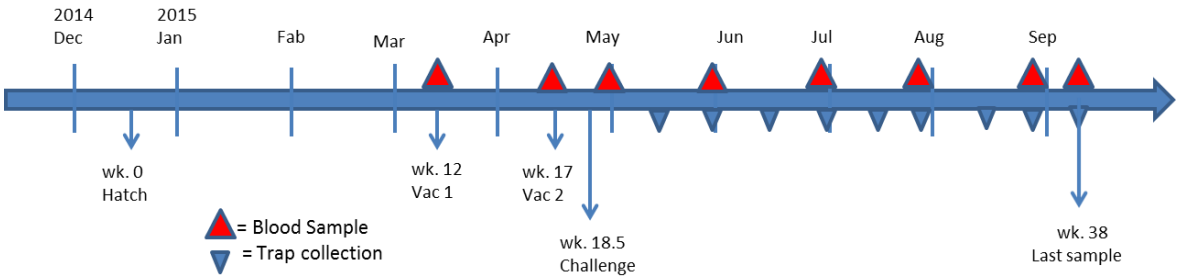

Figure 4.2 Poultry red mite experimental time line.
Time line showing placebo vaccinations and the time course of the Poultry Red Mite (PRM) challenge experiment. The timings of PRM trap counts (below the line) and blood sample collections (above the line) are also indicated.

4.2.2 ELISA Assay

The ELISA assays for chicken APP were obtained from Life Diagnostics Inc (Section 2.2). They were performed according to the manufacturer's instructions with a dilution factor for the serum samples of 1:10000 for AGP (section 2.2.1.1), 1:20 for SAA (section 2.2.1.2) and 1:40000 for CP (section 2.2.1.3). In brief, two samples from each time were run in one plate and tested against the provided standard, so in total, one plate of ELISA is used for running to test 37 samples in addition to two samples for high and low quality control obtained from vaccination study (Chapter 3).

For each of the 8 sampling time points equal aliquots of serum from 8 randomly selected birds (n=8) were pooled for SDS-PAGE. Prior to separation by SDS-PAGE gel, the total protein concentration in each pooled sample was determined using a Bradford reagent (Sigma-Adrich, Dorset, UK), with bovine serum albumin standard according to the manufacture's instructions (section 2.2.5, chapter 2). Pooled serum samples were then diluted in milli Q water and mixed 1:1 with Laemmli sample buffer (Bio-Rad, USA) 5% (v/v) of ß-mercaptoethanol, to achieve a final serum protein concentration of 2 mg/ml. This mixture was then heated at 95oC for 4 minutes. Then 10 µl of each heated sample (20 µg protein/ well) as well as pre-stained protein ladder (17-250 kDa, PageRuler prestained protein ladder, Thermo Scientific Inc., USA) were placed into the wells of an 18-well, 4-15% CriterionTMTXGTM precast gel (Bio-Rad, USA). The proteins were then separated in MOPS running buffer (BioRad #161-0788) at 300 V for 20 min. The gels were stained

for 1 h in a colloidal solution of Coomasie brilliant blue stain G-250 dye 0.1 % (w/v), 10 % (v/v) acetic acid, 40 % (v/v) ethanol; (Invitrogen, Manchester, UK). Protein bands were resolved by de-staining using a solution of 10 % (v/v) acetic acid; 25 % (v/v) methanol. An image scan of each gel was then created using a UMAX Power Look III scanner (Hamrick software, USA).

4.2.3 Protein band identification using Nanoflow HPLC Electrospray Tandem Mass Spectrometry (nLC-ESI-MS/MS)

4.2.3.1 Protein band sample preparation:

Eleven selected protein bands (see results section, Figure 4.8) were excised manually by scalpel and placed in individual vials to be subjected to in-gel digestion for protein extraction prior to identification via mass spectrometry analysis. Gel pieces were washed with 100 mM ammonium bicarbonate for 30 minutes and then for a further h with 100 mM ammonium bicarbonate in 50% (v/v) acetonitrile. After each wash all solvent was discarded. Gel plugs were then dehydrated with 100% acetonitrile for 10 minutes prior to solvent being removed and dried completely by vacuum centrifugation. Dry gel pieces were then rehydrated with 10µl trypsin at a concentration of 20ng/µl in 25mM ammonium bicarbonate (Cat No. V5111, Promega, Madison, WI, USA) and proteins allowed to digest overnight at 37^{o}C. This liquid was transferred to a fresh tube (first extract), and gel pieces washed for 10 min with 10µl of 50% acetonitrile. This wash was pooled with the first extract, and the tryptic peptides dried.

Proteins were identified using nanoflow HPLC electrospray tandem mass spectrometry (nLC-ESI.MS/MS) at Glasgow Polyomics. Peptides were solubilized in 2% acetonitrile with 0.1 % trifluroacetic acid and fractionated on a nanoflow uHPLC system (Thermo Scientific RSLCnano) before analysis by electrospray ionisation (Collgros et al., 2013) mass spectrometry on an Amazon Speed ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (Thermo Scientific). Peptides were desalted and concentrated for 4 min on a C18 trap column followed by an acetonitrile gradient (in 0.1% v/v formic acid) (3.2 - 32% v/v 4 - 27 min, 32 % to 80% v/v 27 - 36 min, held at 80 % v/v 36- 41 min and re-equilibrated at 3.2 %) 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 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 and with the FDR set at 0.01.

MS data were processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server. Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI Genbank database, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses and with the Mascot score set off as 100.

4.2.3.2 Protein band densitometry measurements

To determine the protein concentrations in each electrophoretic band of interest, and to allow for individual bird variation, samples from 4 individual birds at each time points (8 time points) were run on 1D SDS-PAGE gels. Each gel contains two set of samples, each set is included one sample from each time point, two gels were required to run the four sets of samples. Two sets of samples in one gel were analysis and another two set samples for the same time point were analysed in the second gel and the average of the four bands were used for the calculation of the average of the band concentration by use imageJ. A digitised image scan of each gel was created using a UMAX power look III scanner (Hamrick software, USA). The concentration of each band was then estimated using ImageJ software (https://imagej.nih.gov/ij/). This software generates a plot profile for the grey levels that represent the relative % density of each band. The area under each peak is then quantified and the % values generated expressed as a percentage of the total. The mean relative % density for each band for n=4 birds were computed and then converted to mg/ml by assuming that the total concentration of protein in each lane was equal to 2mg/ml (the original concentration of the loaded sample), normality tests ran and One-way ANOVA Tukey test have been used to compare each band for eight time points.

4.2.4 Data and statistical analysis

Results obtained were initially stored in Excel (Microsoft 2010) and simple descriptive statistics were obtained. Data were later exported to Minitab statistical software; Minitab 17.1.0 for further analysis.

Tests for normality were run on the acute phase proteins values using normality test to analyse effects on serum APP of PRM challenge in layer hens. The intensity of the protein bands on one D gel was determined by imageJ (NIH-software). P-value was considered significant at <0.05. To analysis if there any significant changes of the protein bands obtained by imageJ, normality tests run and One-way ANOVA Tukey test have been used to compare each band at eight time points to evaluate the effect of PRM infestation on each blood APP by comparing serum samples taken prior to the PRM challenge (weeks 12 and 17) with those taken following the challenge (weeks 19, 23, 27, 31, 36, and 38). Also Mann-Whitney's test have been used to analysis the differences between protein level of bands for weeks 12 and 17.

4.3 Results

4.3.1 Poultry red mite (PRM) infestation levels

Summary statistics for PRM counts made at each sampling time point are provided in Table 4.1 and presented as box plots in Figure 4.3. Low numbers of PRM were recorded in the first traps at week 21 and 23 (Figure 4.3). PRM numbers subsequently increased reaching a peak at week 33. Following the peak, the PRM numbers started to decrease in weeks 36 (late August) and 38 (September).

Table 4.1 PRM counts at populations over time.
Summary statistics showing mean of PRM, letters and columns with different colours are statistically different from one another (P<0.05). Data provided by Dr J Bartley, Moredun Research Institute).

	Experimental week (Hen age)								
	21	**23**	**25**	**27**	**29**	**31**	**33**	**35**	**37**
Total mites	510	1101	4845	18990	40983	46038	56743	38993	20505
Mean	5	11	50	198	436	480	624	410	216
STDEV	6.970	17.708	47.733	127.033	290.378	352.211	388.997	316.126	121.736
Num of traps (n)	95	96	96	96	94	96	91	95	95
SEM	0.715	1.807	4.872	12.965	29.950	35.947	40.778	32.434	12.490
	a	a	a	b	c	c	d	c	b

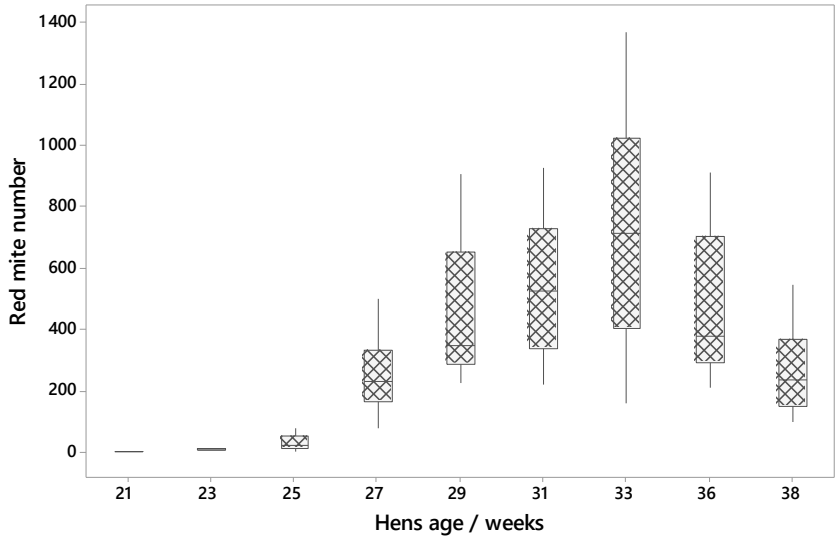

Figure 4.3Changes in PRM population over time.
PRM numbers recorded in traps (n=92) at consecutive sampling time points following a PRM challenge at 18.5 weeks of age. The highest level of PRM infestation was observed at 33 weeks of age which corresponded to late July and early August. Data presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles. (Data kindly provided by Dr J Bartley, Moredun Research Institute).

4.3.2 Serum concentrations of APP and their relationship to PRM infestation rates

4.3.2.1 Serum amyloid- A (SAA)

There was a significant increase in the concentration of SAA in serum obtained post infestation at 27 (P<0.05), 31 and 36 weeks (P< 0.01) compared to that taken prior to the PRM challenge (weeks 12 and 17) (Figure 4.4).

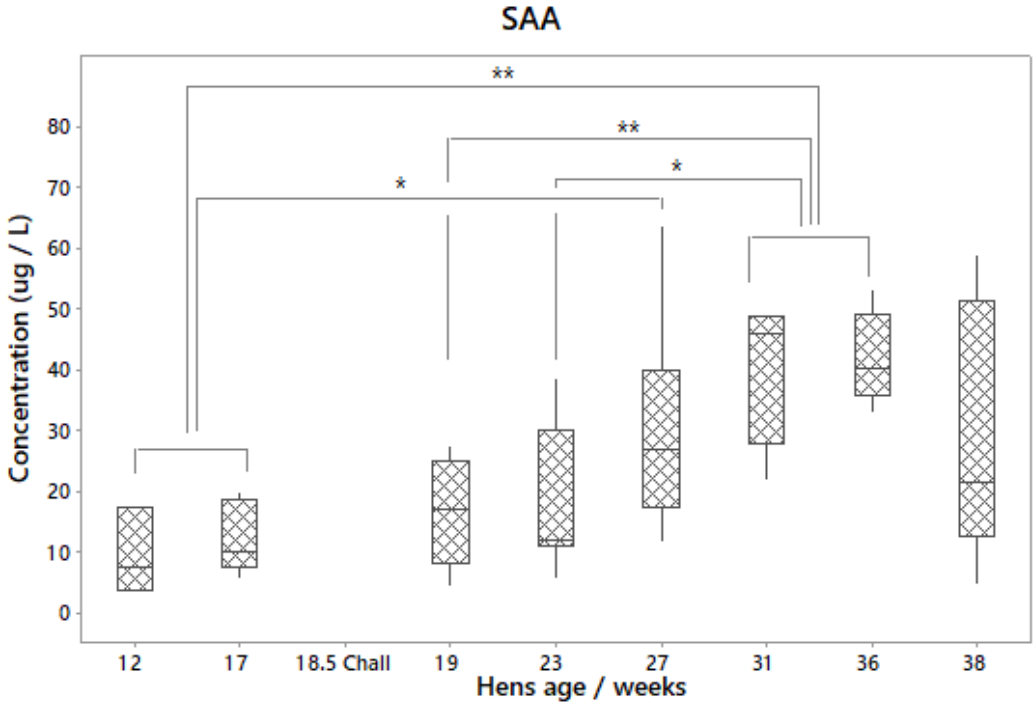

Figure 4.4 SAA response to PRM infestation.
Blood serum levels of SAA pre-and post PRM challenge at 18.5weeks of age. Data (n=115) presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles. Horizontal bars indicate statistical differences between groups (** = P≤0.01, * = P≤0.05).

The pattern of change in the mean SAA levels closely mirrored the mean PRM levels of infestation (Figure 4.4). To investigate this relationship further, data where the PRM infestation levels could be directly matched to a blood sample from the same cage were identified and analysed using Spearman's correlation. This revealed a statistically significant and positive correlation (r^2 = 0.489; P=0.0045) between SAA levels and the numbers of PRM (Figure 4.5).

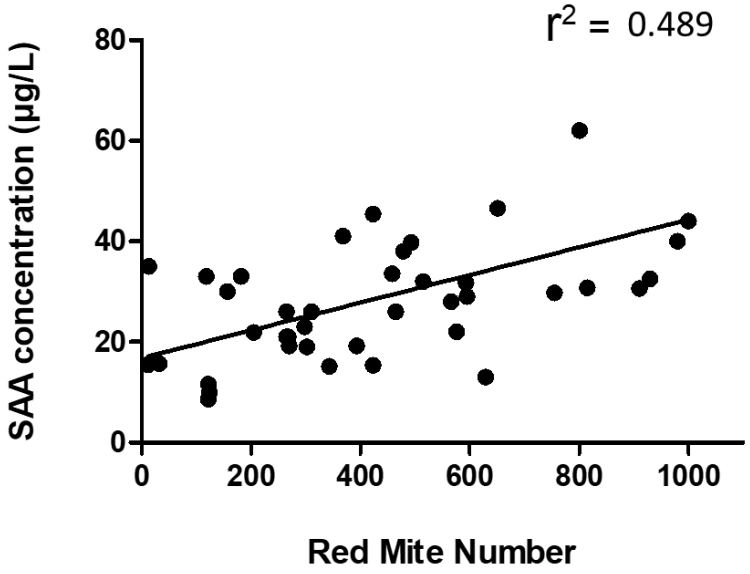

Figure 4.5 Correlation of SAA serum concentrations with PRM numbers.
Scatter plot illustrating the relationship between SAA and PRM (n=96 cages), there was moderate correlation (r^2=0.48) when analysed by Spearman's correlation.

4.3.2.2 Alpha-1 acid glycoprotein (AGP)

There was no consistent change in the serum AGP levels pre-and post PRM challenge. Levels of serum AGP prior to infestation (week 12) were significantly higher than at week 19 ($P<0.05$) and 27 ($P< 0.01$). AGP levels following the PRM challenge were not significantly different than that observed at 17 weeks (Figure 4.4.6). There was no correlation between the AGP concentration and PRM infestation levels ($r^2 = 0.257$; $P < 0.6$).

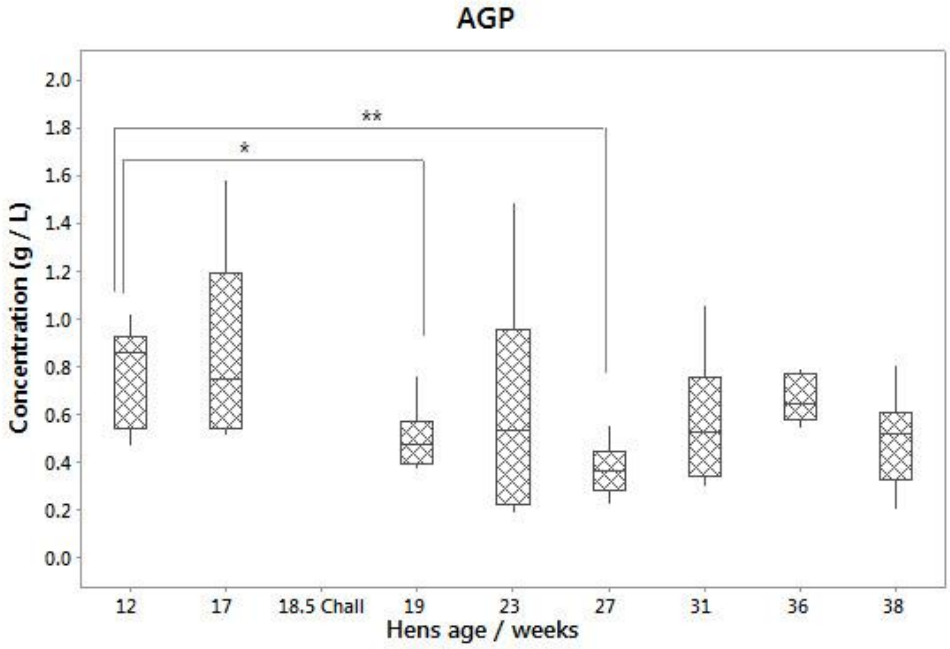

Figure 4.4.6 Response of AGP to PRM infestation.
AGP levels in blood sera pre-and post PRM challenge at 18.5weeks of age. Data (n=15) presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles. Horizontal bars indicate statistical differences between groups (** = P≤0.01, * = P≤0.05)

4.3.2.3 Ceruloplasmin (CP)

Serum concentrations of CP at 12 weeks were significantly higher (P<0.05) than the 17 weeks (pre-challenge). Also, were significantly higher (P< 0.01) than at all time points post-infestation, though not significantly different from the concentration at week 17 (pre-challenge time point) (Figure 4.7). CP levels were not correlated with PRM infestation levels (r = 0.328; P < 0.3).

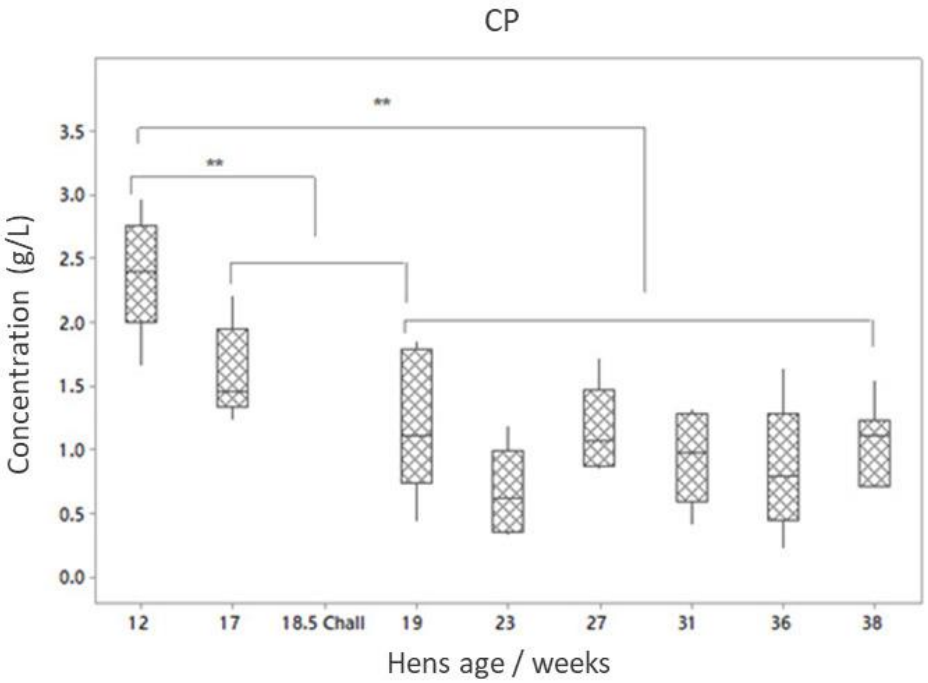

Figure 4.7 CP response to PRM infestation.
CP levels in blood sera pre-and post PRM challenge at 18.5weeks of age. Data (n=15) presented as box and whisker plots with median in the box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles. Horizontal bars indicate statistical differences between groups (** = P≤0.01, * = P≤0.05).

4.3.3 Identification of the serum protein components

SDS PAGE separation of the pooled serum samples from all sampling points are compared in Figure 4.8. A full detail of the proteomic analysis is provided in Appendix C. Two high molecular weight (MWt) bands of approximately 300 kDa (band 1) and 250 kDa (band 2) were absent in the 12-week sample but present in all of the other samples. The 300 kDa protein (band 1) corresponded to Gallus gallus apolipoprotein-B precursor (gi|113206052) with coverage 40%. The 250kDa protein (band 2) corresponded to Gallus gallus vitellogenin-2 (gi|71896765) with coverage of 66%. Bands 3 and 5 with MWt of ~200 and 100 kDa both corresponded to the same protein (alpha-2 macroglobulin like protein). Ovotransferrin, Albumin, complement C, immunoglobin gamma, Apolipoprotein A-IV and Apolipoprotein A-l were amongst the other proteins identified. Notably, albumen was predominant in several bands.

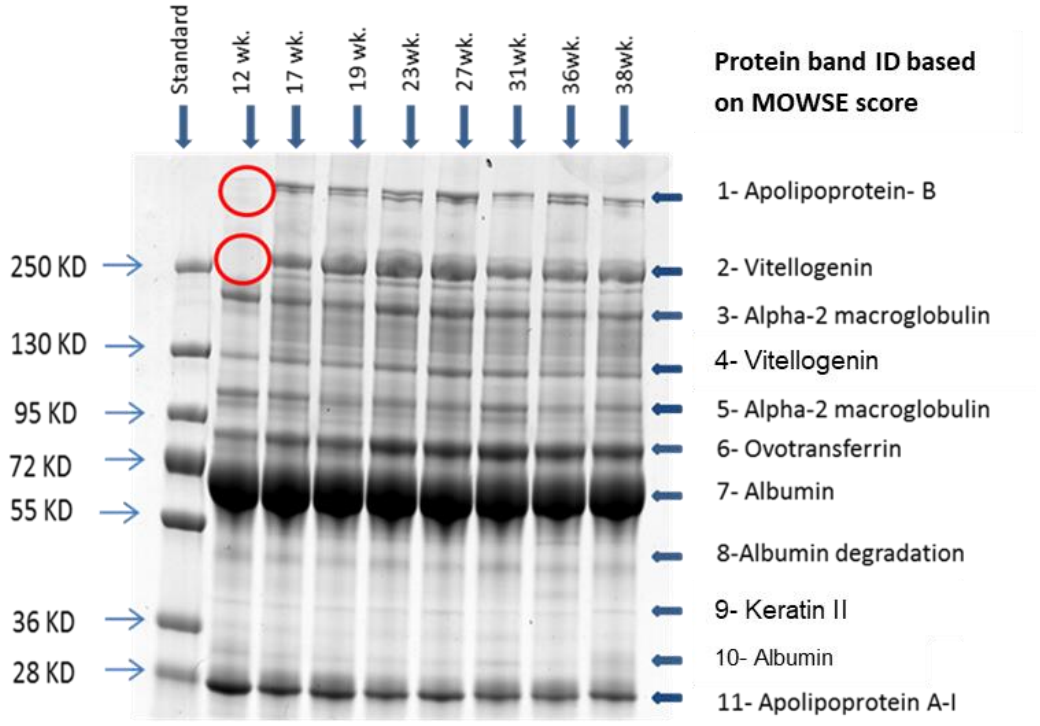

Figure 4.8 Electrophoretic profiles of hens at age 12-38 weeks.
Electrophoretic profiles of pooled serum samples (n=15) corresponding to different sampling time points. Two high molecular weight bands are missing in the serum sampled at week 12 (red circles). Subtle differences in the density of the other protein bands can also be observed. The protein identity of each band based on the MOWSE score is indicated (further details are provided in Table 1 and in the supplementary data). The protein identity of each band is indicated (further details are provided in appendix 4.1.

4.3.4 Protein Densitometry Measurements

Figure 4.4.9 compares the relative concentration of proteins in bands 1-11 between 12 and 17 weeks of age for four individual hens selected at random at each time point. As well as there being a significant increase in Apolipoprotein-B (MWt ~300 kDa; P = 0.007) and Vitellogenin-2 (MWt 250 kDa; P = 0.007) there was a significant decrease in Alpha-2 macroglobulin's (~200 kDa, band number 3; P = 0.007 and ~100 kDa, band number 5; P = 0.007) and Apolipoprotein A-1 (28 kDa; P = 0.003) between 12 and 17 weeks of age.

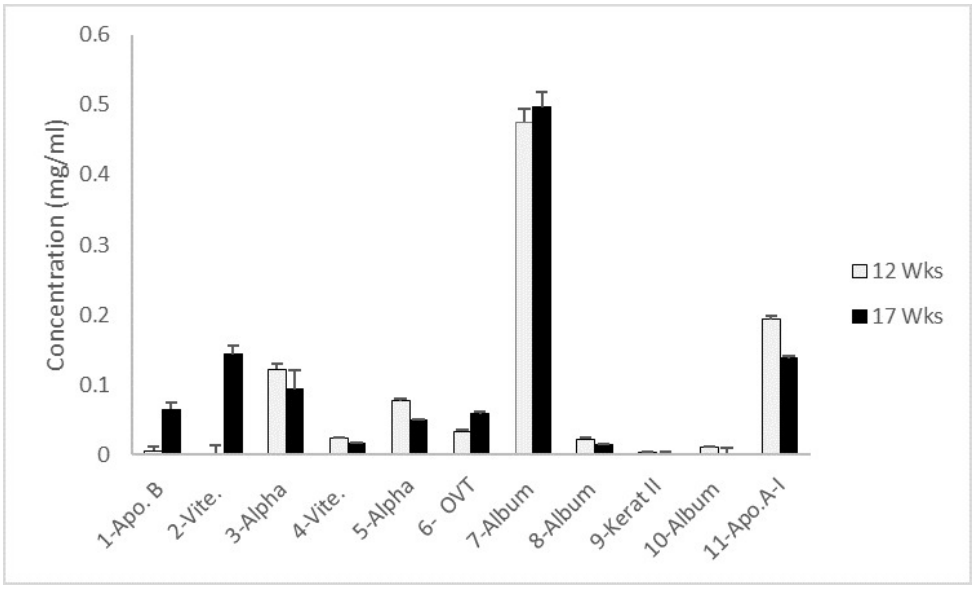

Figure 4.4.9 Comparison of the relative concentration of hen serum proteins.
Mean ± standard deviation protein band densitometry measurements (mg/ml) for n=4 individual hens at 12 and 17 weeks of age. Significant differences were observed for Apolipoprotein-B (band 1-Apol-B; P = 0.007), Vitellogenin-2 (band 2-Vite.; P = 0.007), Alpha 2-macroglobulin (band 3-Alph: P = 0.007 and 5-Alph: P = 0.007), and Apolipoprotein A-I (band 11-Apol. A-I; P = 0.003) normality tests ran and Mann Whitney comparisons made.

Protein densitometry measurements carried out on serum from four individual hens at each sampling time point pre and post PRM challenge revealed that Apolipoprotein-B levels increased 22.6-fold (from 0.008 mg/ml to 0.181 mg/ml; $P \leq 0.01$) and Vitellogenin-2 increased 100-fold (from 0.002 mg/ml to 0.20 mg/ml; $P \leq 0.01$) between 12 and 23 weeks of age and remained high throughout the study period (Table 4.2). Ovotransferren (band 6) increased significantly by 1.9 fold (from 0.107 to 0.213 mg/ml, $P<0.05$) at week 36 compare to week 12. Meanwhile Alpha-2 macroglobulin (band 5) and Apolipoprotein A-I (band 11) decreased by 1.9 fold (from 0.139 to 0.070 mg/ml, $P<0.05$), and 2.1-fold (from 0.193mg/ml to 0.09 mg/ml, $P< 0.05$) respectively at 23 weeks compared to week 12. Other protein bands were also compared but no statistically significant differences were observed other than that for Alpha-2 macroglobulin (band 3 and 6) between 12 and 17weeks(Table 4.2)

Table 4.2 Protein densitometry measurements compared across all time points.
Data are presented as median and range values (n). Meadian not sharing the common superscripts are significantly different (P ≤ 0.05) One way ANOVA Tukey test used to analysesd significat difference of bands densitometric throughout the expermint.

Age (weeks)	Apolipoprotein B (band 1)	Vitellogenin-2 (band 2)	Alpha-2 macroglobulin (band 3)	Vitellogenin-2 (Band 4)	Alpha-2 macroglobulin (band 5)	Chain A Ovotransferrin (Band 6)	Serum Albumin (Band 7)	Serum albumin Precursor (Band 8)	*Keratin, type II* (Band 9)	Serum albumin Precursor (Band 10)	Apolipoprotein-A-I (band 11)
12	0.006 (0.001-0.006) a	0.002 (0.001-0.006) a	0.156 (0.104-0.197) a	0.057 (0.054-0.069) a	0.139 (0.102-0.174) a	0.107 (0.104-0.113) a	1.076 (1.072-1.090) a	0.026 (0.022-0.036) a	0.066 (0.003-0.008) a	0.006 (0.0005-0.007) a	0.43 (0.37-0.45) a
17	0.078 (0.035-0.089) b	0.109 (0.080-0.176) b	0.075 (0.066-0.098) b	0.060 (0.056-0.061) a	0.071 (0.054-0.098) b	0.150 (0.137-0.156) b	1.051 (1.039-1.155) a	0.022 (0.016-0.025) a	0.005 (0.001-0.007) a	0.006 (0.004-0.013) a	0.32 (0.29-0.33) b
19	0.065 (0.061-0.073) b	0.149 (0.115-0.176) bc	0.082 (0.057-0.107) b	0.042 (0.035-0.055) a	0.089 (0.063-0.114) ab	0.175 (0.161-0.198) b	1.052 (1.035-1.139) a	0.023 (0.019-0.028) a	0.004 (0.003-0.007) a	0.003 (0.001-0.004) a	0.29 (0.23-0.35) bc
23	0.116 (0.086-0.164) c	0.220 (0.177-0.285) bc	0.065 (0.056-0.087) b	0.057 0.055-0.065) a	0.074 (0.049-0.109) b	0.17 (0.161-0.198) b	0.993 (0.983-1.005) a	0.028 (0.017-0.033) a	0.007 (0.003-0.009) a	0.003 (0.001-0.004) a	0.24 (0.23-0.25) bc
27	0.115 (0.099-0.120) bc	0.206 (0.183-0.250) bc	0.063 (0.053-0.094) b	0.045 (0.035-0.070) a	0.070 (0.040-0.157) ab	0.197 (0.171-0.225) c	1.044 (0948-1.072) a	0.020 (0.013-0.027) a	0.005 (0.002-0.008) a	0.006 (0.004-0.013) a	0.22 (0.19-0.24) c
31	0.092 (0.060-0.092) bc	0.278 (0.131-0.336) c	0.079 (0.047-0.084) b	0.058 (0.048-0.069) a	0.077 (0.061-0.085) b	0.207 (0.192-0.225) c	0.964 (0.930-0.987) a	0.026 (0.019-0.051) a	0.005 (0.003-0.009) a	0.007 (0.002-0.010) a	0.22 (0.16-0.27) c
36	0.107 (0.101-0.122) bc	0.189 (0.159-0.300) bc	0.072 (0.043-0.081) b	0.056 (0.036-0.061) a	0.084 (0.066-0.010) ab	0.213 (0.168-0.988) c	0.956 (0.935-0.967) a	0.028 (0.020-0.030) a	0.006 (0.004-0.008) a	0.003 (0.002-0.003) a	0.27 (0.23-0.29) bc
38	0.085 (0.068-0.131) b	0.163 (0.156-0.192) bc	0.068 (0.065-0.074) b	0.061 (0.055-0.060) a	0.084 (0.067-0.110) ab	0.195 (0.159-0.240) c	1.020 (0.981-1.050) a	0.030 (0.023-0.049) a	0.004 (0.002-0.004) a	0.008 (0.002-0.17) a	0.26 (0.21-0.30) bc

4.4 Discussion

Infestation of laying hens with PRM resulted in an APP response which was characterised by SAA concentrations being 6.25 fold higher in the blood sera at 31 and 36 weeks compared to week 17 pre-infestation levels ($P<0.01$). Mite infestation has been reported to cause a significant increase in SAA in other species. For example, experimental infestation of sheep with the sheep scab mite (*Psoroptes ovis)* led to a significant elevation of SAA ($P \leq 0.001$) compared to pre-infestation levels (Wells, et al., 2013). A similar SAA response has also been reported in the case of mange infestation (*Sarcoptes scabiei)* in the Alpine ibex (Rahman et al., 2010), with an SAA peak occurring at week 4-5 post infestation. The serum concentration of SAA in the current study was moderately correlated to the PRM infestation levels ($r^2 = 0.4891$; $P=0.004$), suggesting that exposure to PRM may trigger an APP response probably mediated by cytokines released by the cells of the innate immune system in response to the mites feeding (Kowalski and Sokol, 2009). However, as the correlation coefficient $r^2=0.489$, it does mean that the SAA is responsible for only ~ 50% of the variation but it would be expected that there are many other factors involved in development of the PRM count such as environmental conditions, acquired as well as innate immune responses and nutrition. APP can also respond to increased corticosterone (Zulkifli et al., 2014), which has previously been shown to rise in response to PRM infestation in laying hens (Kowalski et al., 2006). The release of glucocorticoid alters immune function by downregulating inflammatory cytokines and up-regulating anti-inflammatory cytokines (Sheridan et al., 1994; Elenkov and Chrousos, 2002). Monitoring SAA levels could therefore be valuable as an aid to assessing the PRM infestation level and the welfare of laying hens in poultry farms as a means to monitor the innate immune response mediated via pro-inflammatory cytokines (Baumann and Gauldie, 1994; Jensen and Whitehead, 1998; O'Reilly and Eckersall, 2014).

Neither AGP nor CP levels increased in response to the PRM infestation. The pro-inflammatory cytokine stimulation of the acute phase response was therefore relatively moderate as it did not stimulate the production of these two APP. It has been noticed in the vaccination study (chapter three, section 3.3.4) that the SAA was more sensitive compared to other APP and that it increased by 2.5-fold at 24h post vaccination. In other species, SAA is one of the most sensitive major APP to stimulation (Ceron et al., 2005; Ceciliani et al., 2012). Consequently, a

similar immunological reaction might be true for laying hens. In future studies it would be useful to monitor the changes in pro-inflammatory cytokines such as interleukin 1 and interleukin 6, to confirm that only a moderate cytokine response had been stimulated.

The serum concentrations of AGP and CP fell in the early stages of this study (Figure 4.7). Such subtle changes may reflect an age-related alteration in these two APP; age related differences in APP have been reported in other species such as in pig with reduction of AGP taking place over the first weeks post-parturition (Christoffersen et al., 2015), but until now have not been reported in laying hens. The fall in CP between 12 and 19 weeks of age for example may be associated with the onset of sexual maturity and oogenesis. Oocyte development requires the presence of various elements, including copper that binds specifically to egg proteins such as Ovotransferrin (Johnson, 2015). CP contains over 95% of the copper found in the plasma (Martinez-Subiela et al., 2007). The high demand for copper associated with follicle development at the onset of lay therefore provides a plausible explanation for the observed reduction in serum CP levels in our study.

Additional changes in the serum proteins at the onset of lay were investigated by SDS-PAGE gel electrophoresis of 4 individual samples from each time point run on electrophoresis and analysed by densitometry. Both Apolipoprotein-B (MWt 300 kDa) and Vitellogenin-2 (MWt 250 kDa) were significantly increased by 17weeks and reached a peak by 23weeks which is just in advance of peak production for Lohmann brown layers. These results were obtained by densitometry of serum electrophoresis, a standard procedure in veterinary diagnostic laboratories (Eckersall, 2011), but which is not specific, and it would be of value in future investigation to apply quantitative methods of greater specificity such as immunoassay or quantitative proteomics. However, the results obtained here indicate the large increases in apolipoprotein-B and vitellogen-2 at week 17 and also the time of their maximum level in chicken serum. Both Apolipoprotein- B and Vitellogen-2 are lipid-binding proteins and are precursors of the major proteins of the yolk (Finn, 2007). At sexual maturation, and in response to rising levels of estrogen, plasma levels of ovotransferrin Vitellogenin have been shown to rise as production in the liver increases (Wallace, 1985; Johnson, 2015). Ovotransferrin is a major avian egg white protein constitutes 12 to13% of total egg white (Stadelman and Cotterill, 1986; Wu and Acero-Lope, 2012). In the current

study, the significant increase of OVT in blood chickens by 31 and 36 weeks compared to 12 weeks that could related to the level of PRM infestation. It is well known that anemia is one of the significant deleterious effect of PRM on their avian host (Chauve, 1998; Kilpinen, 2005). This anemia increase in severity with the increase of the PRM infestation rate. Consequently, it could be iron diffeciency in anemic chickens is the reason to increase OVT level in blood which combined a beak level of PRM infestation (Figure 4.3). As some researchers have observed an increase in mRNA in chickens on iron deficient diets (Cochet et al., 1979; McKnight, et al., 1980).In the ovary, vitellogenin is taken up by the developing oocytes to form lipovitellin, phosvitin and yolk plasma glycoproteins (Hiramatsu et al., 2002). In addition, vitellogenin (and albumin) are important in the transport of circulating non-diffusible protein bound calcium, which is important for eggshell formation (Johnson, 2015). Liou and co-workers (2007) reported that vitellogenin levels in the plasma were positively correlated to egg productivity in a local breed of chicken in Taiwan (TRFCC). It would be of interest to determine if a similar relationship exists in more highly selected commercial breeds of layer chickens.

Oogenesis is also considered to be the main factor affecting the plasma levels of apolipoproteins and total lipid in chicken plasma, thus enabling efficient delivery of very low-density lipoproteins (VLDL) to the egg (Pinchasov et al., 1994). Apolipoprotein B is a major protein of VLDL. This protein undergoes proteolytic cleavage in the yolk into at least nine protein fragments, the presence of some of which have been shown to contribute to the excellent emulsifying properties of egg yolk (Jolivet et al., 2008). Apolipoprotein A-I is involved in incorporating lipid into the yolk mass (Vieira et al., 1995), so the demand for this protein will also be greatest at peak production (Finn, 2007). Interestingly Liou and his co-workers (2007) found that the levels of Apolipoprotein A-I were negatively correlated to egg productivity in their study of a native Taiwan breed of chicken.

Alpha-2 macroglobulin (bands 3 and 4, Figure 4.4.9) significantly decreased between 12 and 17 weeks and then remained at a relatively constant level for the remainder of the experiment. This protein is a part of the innate immune system and functions to clear active proteases from tissue fluids (Armstrong and Quigley, 1999). Alpha-2 macroglobulin also known as Ovostatin has been isolated from the plasma of vertebrates as well as the egg white of birds and reptiles (Armstrong

and Quigley, 1999; Rehman et al., 2013). A decrease in plasma levels of this protein at the onset of lay may be related to oviduct development and the increased demand for this protein to be incorporated into the forming egg. Previous studies on human plasma samples have reported that the MWt of Alpha-2 macroglobulin is 180 kDa and 85 kDa (Harpel and Brower, 1983). In the current study, the MWts were ~200 and ~100 kDa (Figure 4.8). This could be related to differences in the degree of glycosylation, species variance, or methodology differences.

In relation to the third aim of this study, which was to determine if PRM significantly alters the serum protein profile of laying hens, we did not find any differences in the high abundance serum proteins which could be directly atributable to the PRM challenge when the serum proteins were separated using SDS-PAGE (Figure 4.8). Changes in SAA were only observed using an ELISA which had the sensitivity to measure this low abundance protein. SAA has a low molecular weight (14 kDa) when it becomes dissociated from the high-density lipoprotein and is known to be a difficult protein to analyse with SDS-PAGE procedure (Soler et al., 2011). This, together with SAA having a concentration in the μg/L range (>1,000 lower than high abundance proteins in serum such as albumin and IgG which have concentrations of 1-50 g/L) means that electrophoresis is not suitable for monitoring this acute phase and low abundance protein.

In conclusion, following a PRM challenge at 18.5 weeks, the serum levels of SAA were significantly increased, and this was subsequently found to be positively correlated with the level of PRM infestation. In this study proteomic investigation revealed a marked change in the levels of two major proteins viz. Apolipoprotein B and Vitellogenin-2 in the sera of hens between 12 and 17 weeks of age. The serum proteome however did not appear to be significantly altered in the weeks following the PRM challenge. It may be that the levels of mite infestation were too low to stimulate a major APR or that the chronicity of the disease did not show a clear proteomic change as the hens quickly acclimatized to the stress of the infestation. Further work is needed to establish if serum proteins differ in high and low producing hens and to determine if PRM infestation alters the serum protein profiles in older birds.

Chapter 5 Effect of *E. coli* endotoxin on the acute phase proteins and associated blood components in broiler chickens.

5.1 Introduction

E. coli is a Gram-negative pathogenic bacterium that can have a significant effect on poultry production (Kemmett et al., 2014). Lipopolysaccharide (LPS) is an endotoxin presented as part of the outer membrane component of Gram negative bacteria like *E. coli* (Zimecki et al., 2004). LPS is the main inducing factor for endotoxemia and its consequential immune response (Salomao et al., 2012). An LPS extract can therefore be used in challenge experiments without the need to use infectious agents that are difficult to control without appropriate isolation facilities (Koppenol et al., 2015).

The LPS triggered immune activation leads to the production of inflammatory mediators (cytokines and chemokines) (Haudek et al., 2003; Skarnes et al., 1981), which influence the blood protein profile, including acute phase proteins (APP) (Saloma et al., 2012). The acute phase response (APR) can also be trigger by different stressors, such as environmental, pathogenic or a management disorder. Such stressors also induce an activation of the sympathetic-adreno-medullary (SAM) and hypothalamic–pituitary–adrenal (HPA) axes and result in the release of catecholamines and glucocorticoids, respectively as detailed in chapter 1 (Figure 1 .1) (Dickens et al., 2010; Mormede et al., 2007). In general, the aim of the stress response is to maintain or re-establish the homeostatic state, and sustain vital physiological activities (Zulkifli et al., 1995).

Several studies related to stress physiology of poultry have emphasised that corticosterone is a major hormone of the HPA axis, in chickens (Ohtsuka et al., 1995). Corticosterone plays a multifunctional role through alteration of endocrine and metabolic factors, including leukocytes and immune mediators (Shini et al., 2008). In addition, corticosterone increases significantly within minutes after the introduction of a stressor (Jones et al., 2016); therefore, its blood concentration is widely used as an indicator of stress (Khansari et al., 1990; Husband,1993). Induction of adrenocortical activity is known to generally precede heterophilia and lymphopenia (Gross and Siegel, 1983; Maxwell, 1993). However, it has also been reported that raised H/L ratios in chickens subjected to various stressors, can occur independently of plasma levels of corticosterone (McFarlane and Curtis, 1989). Plasma corticosterone levels and H/L ratios therefore represent two distinct events, namely the short term and longer-term responses to stress in birds

(Shini et al., 2007). In the context of this chapter it is therefore hypothesised that following an LPS challenge, corticosterone levels will increase in advance of any change in the H/L ratio. Packialakshmi, et al. (2016) showed that a *Salmonella* derived LPS injection caused AGP, the best characterised chicken APP, to increase in layer chickens after 24 h. A major limitation of this study however was that these authors only sampled blood from the treated birds once viz. 24 h post injection. One objective of the experiment described in this chapter was to characterize how corticosterone, H/L ratios and APP change over 72 h in broiler chickens following an LPS challenge.

Birds and mammals appear to respond to an LPS challenge in a similar way in that there is an increase in production of cytokines and antibodies and the hemodynamic responses are also similar (Sunwoo et al., 1996; Nakamura et al., 1998 a, b; Weining et al., 1998; Rautenschlein et al.,1999; Xie et al., 2000; Wang et al., 2002). Tolerance to LPS is greater in birds than in mammals (Smith et al., 1978; Adler and DaMassa, 1979; Roeder et al., 1989). To date however there is relatively limited information is available on systemic APP responses to LPS in birds (Smith et al., 1978; Xie et al., 2000). APP are mainly produced by hepatocytes in the liver, though recent evidence suggests that many other tissues express APP even in healthy animals, albeit at significantly lower concentrations, and most interestingly in the absence of a systemic APR (Marques et al., 2017). In order to study the proper utilisation of APP as biomarkers during disease, it is essential to understand where precisely APP are being produced and secreted (Lecchi et al., 2009). Wieland et al., (2016) hypothesised that APP derived from the liver have a systemic protective role, whereas, extrahepatic tissue production of APP may be important for local protection and repair (Schroedl et al., 2016) and may also have different molecular characteristics (Inforzato et al., 2006). In other words, locally and hepatically produced APP could represent distinguishable proteo-forms according to the signals and capabilities of the expressing cells (Schroedl et al., 2016).

In healthy chickens, extrahepatic production of APP mRNA has been detected in a range of tissues including the gastrointestinal track, the respiratory and lymphatic systems (Marques et al., 2017), but no information is currently available relating to expression levels under disease or challenge conditions.

The aim of the research described in this chapter was to subject broiler chickens to an LPS *E. coli* challenge and to measure and compare the plasma levels of corticosterone, the H/L ratio and the systemic plasma levels of three APP (AGP, SAA and OVT) over a time course of 72 h (six times). A further aim was to use real-time qPCR to compare the mRNA expression levels of these APP in the liver and in a range of extrahepatic tissues in control and challenged birds). The objective here was to determine if the LPS challenge had induced both a systemic and a local immune response. A limitation of this study was that tissue samples could only be harvested at 72 h post challenge.

5.2 Materials and methods

5.2.1 Chicks and Housing

This trial was conducted at Glasgow universities research farm at Cochno, near Glasgow. Sixty, day old ROSS 308 broiler chicks were obtained from a commercial supplier (PD Hook (Hatcheries) Ltd, Dunbar, UK) and transported to the farm. On arrival, the chicks were fitted with unique wing tags and placed in one of 4 pens in a controlled environment room. Initially a brooding lamp and ring was placed in the centre of each pen along with supplementary feeders and drinkers. Each pen had a concrete floor covered with 2-3 inches of wood shavings. The total dimension of each pen was 3 m × 2.2 m. A commercial chick crumb was made available ad libitum, and the lighting and temperature in the room adjusted as per the management guide recommendations. From day 2-15, one pen of chicks per day were handled and moved between their pen and the adjoining prep room. At day 15 the number of chicks in each pen was adjusted from 15-12 to account for early deaths in some of the pens. From day 15, until the end of the research experiment described below, room temperature was maintained within the thermal neutral zone at 18°C (range 18.0–18.3 during filming) and a 20 h:4 h light: dark cycle was employed. The research experiment was conducted under Home Office Authority under Project and Personal Licences held by the University of Glasgow.

5.2.2 Experimental Design

The research experiment commenced when the chicks were 15 days old. A time 0, the cloacal temperature of all birds was measured by placing a digital thermometer probe carefully into the cloaca (ET402, Libra Medical Ltd, Berks, UK). Approximately 1.2 ml of blood was collected from the wing vein of each bird using heparinized tubes. The birds from pens 1 and 3 (LPS challenge group, total n=24) each then received a subcutaneous injection (SC) of *E. coli* lipopolysaccharide (LPS) (L2630 from Sigma-Aldrich, Dorset, UK) at a dose of 2mg/kg body weight (~ 0.5 ml). The birds from pens 2 and 4 (saline control group, total n=24) each received a SC injection of sterile saline (~ 0.5 ml) as a placebo.

Following administration of LPS or Saline, cloaca temperature and blood sampling was repeated at 6, 12, 24, 48, and 72 h post treatment (PT). All blood samples

were centrifuged (3000 × g) for 15 min at 4 ◦C and the plasma immediately frozen at -20ºC. Blood smears were also prepared at 24, 48 and 72 h. The experiment was terminated at 72 h PT, and all birds were culled by intravenous administration of a lethal dose (1.5-2ml/bird) of Euthatal (200mg/ml, Merial Company). Tissues samples (liver, lung, adipose tissue and gizzard) from saline control (n=6) and LPS challenged (n=9) birds were placed in RNAlater (AM7020, Thermo, Loughborough, UK) and stored at 80 ºC for quantitative real time qPCR.

5.2.3 Heterophil / Lymphocyte Ratios

The H/L ratio in blood sampled from the Saline control and LPS challenged birds was determined at 24, 48 and 72 h. The H/L ratio was measured as described in section (3.2.4).

5.2.4 Corticosterone estimation

Corticosterone was measured in plasma samples, following solvent extraction, using a commercial ELISA kit (Cayman chemical, Ann Arbor, <I, USA, 501320) (Apfelbeck et al., 2017). Extraction efficiency was determined by spiking samples with 10ul H^3-labelled cortisol (Lot. 3632075, Perkin Elmer, Boston Massachusetts, USA). Briefly 50 µl of plasma from each sample was aliquoted into a glass 20 ml tube and mixed with 10 µl of radio labelled Cortisol. and incubated overnight at 4ºC. 5ml of diethyl ether was then added to each tube and shaken for 10 minutes on a mechanical shaker. The solvent phase was decanted into a fresh glass tube following freezing of the aqueous phase with a methanol dry ice bath. Extracts were then evaporated to dryness in a ventilated hot block. Samples were reconstituted in 600ul of the ELISA buffer provided in the kit, by shaking for 1h at RT and storage at 4ºC overnight.

The ELISA was run as per manufacturer's instructions. Briefly, a serial dilution of the standard was prepared S1 (5000pg/ml), S2 (2000 pg/ml), S3 (800pg/ml), S4 (320 pg/ml), S5 (128 pg/ml), S6 (51.2pg/ml), S7 (20.5 pg/ml), and S 8 (8.2 pg/ml). Duplicate aliquots of the standard and samples (50 µl) were added to the plate. 50 µl of the corticosterone AChE tracer was added to each well except for the blank wells. 50 µl of ELISA Antiserum was added to each well except the blank, nonspecific binding, and maximum binding wells. The plate was then covered and incubated overnight at 4ºC. Prior to development of the plate it was washed five

times using the supplied wash buffer, then 200 µl of Elmans reagent provided by the kit was added to each well. The plate was then covered and kept in the dark, at room temperature until the absorbance read using a spectrophotometer at a wavelength of 405 nm in the maximum binding wells averaged between 0.3-1.5 A.U (blank subtracted), typically ~120 minutes. Absorbance results were calculated by subtracting the average absorbance of the blank wells from the standards and samples. The concentrations of Corticosterone were calculated using Assayzap software (Cambridge UK) and corrected for the extraction, reconstitution and percentage recovery (average ±SD, 71.27±30.9)

5.2.5 Acute Phase Proteins

5.2.5.1 ELISA for AGP and SAA

The ELISA assays for AGP and SAA were obtained from Life Diagnostics Inc (Section 2.2). They were performed according to the manufacturer's instructions with a dilution factor for the serum samples of 1:10000 for AGP (section 2.2.1.1), and 1:20 for SAA (section 2.2.1.2) (section 2.2.1.3). Each individual sample was run in duplicate, in brief, two samples from each time were run in one plate and tested against the provided standard, so in total, one plate of ELISA is used for running to test 37 samples in addition to two samples for high and low quality control obtained from vaccination study (Chapter 3).

5.2.5.2 Radial immunodiffusion (RID) for OVT.

Levels of OVT were assessed by RID using specific antibody for chickens OVT as described in section (3.2.7). Each individual sample was run in duplicate.

5.2.6 Expression of AGP, SAA and OVT mRNA in the liver, and selected extrahepatic tissues

5.2.6.1 Introduction to real-time quantitative PCR (qPCR)

A major breakthrough in DNA / cDNA quantification and analysis of gene transcription was achieved through the development of real-time PCR or quantitative PCR (qPCR) (Higuchi et al., 1992; 1993; Zemanick et al., 2010). qPCR allows data collection throughout the PCR process as it occurs, combining amplification and detection into a single step (Wong and Medrano et al.,2005). This is achieved using different fluorescent chemistries such as SYBR® Green which correlate PCR product concentration to fluorescence intensity (Higuchi

et al., 1993). A qPCR reaction may be performed using primers unique to each region to be amplified and tagged with fluorescent dyes.

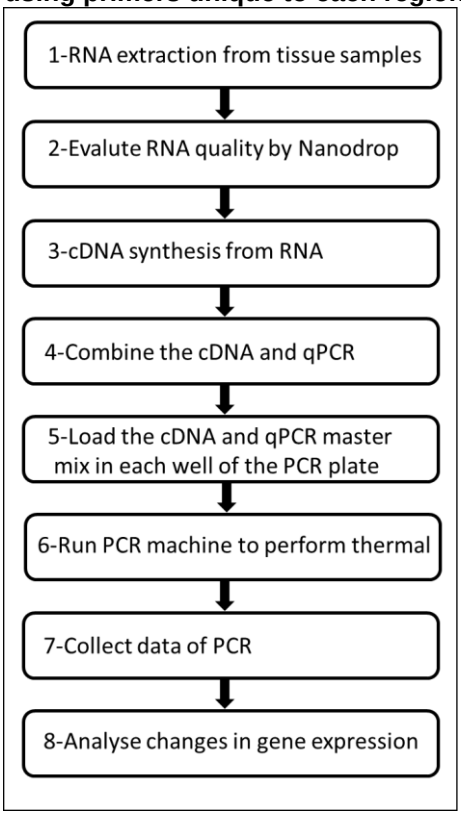

Figure 5.1 shows the general qPCR array procedure for SYBR® Green. The protocol starts by extracting RNA and assessing its quality and concentration by using Nanodrop UV-Vis spectrophotometer (NanoDrop® ND-1000) and ND-1000 V3.1.2 software. Only high-quality RNA samples are converted into PCR template (cDNA) using a reverse transcriptase enzyme. The template is then combined with a ready-to-use qPCR Master Mix, and equal aliquots of this mixture are added to each well of the same PCR array plate already containing gene-specific primer sets. The thermal cycling is performed, and the instrument's software is used to calculate the threshold cycle (Ct) values for all the genes on each PCR array. Finally, the fold changes in gene expression for pair-wise comparison are calculated using the ΔCt method (Palmer et al., 2003).

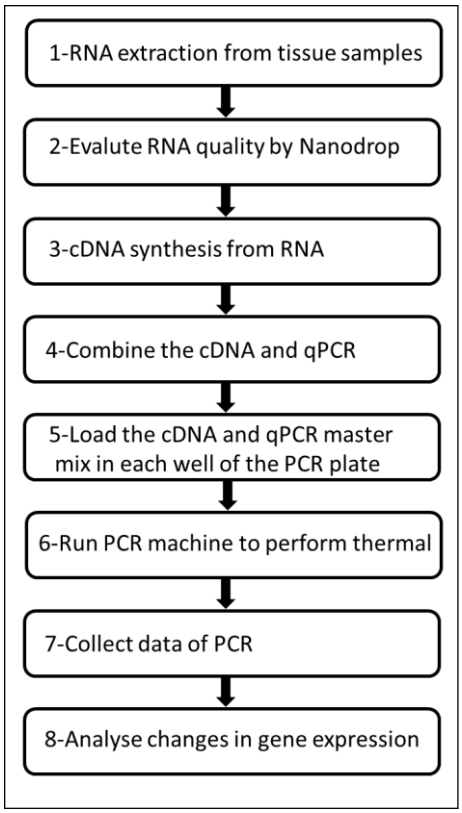

Figure 5.1 General qPCR array procedure.
A scheme of qPCR array procedure for SYBR® Green. High-quality RNA samples are required for analysing gene expression using qPCR arrays.

In the SYBR® Green qPCR method, individual reactions are characterised by the cycle at which fluorescence first rises above the threshold, which is referred to as the Quantification Cycle threshold (Ct). If the amount of target DNA or cDNA in the sample is abundant, amplification is observed in earlier cycles, and the Ct is lower. If the starting material is uncommon, amplification is observed in later cycles, and the Ct is higher. This correlation between fluorescence, Ct, and amount of amplified product enables quantification of the template over a wide dynamic range (Heid et al., 1996).

PCR product accumulates exponentially in the early stages of a reaction and the high-precision nature of the reaction during that time makes the data very reliable. As PCR progresses, the polymerase enzyme become less efficient, the nucleotides and primers concentration diminish; there is an increasing number of new template molecules after each passing cycle that are competing for the reduced amounts of the primers, nucleotides, and polymerase. Thus, the reaction enters the linear phase in which there is great variability with respect to reaction efficiency. Finally, in the plateau phase, there is little, if any, product

accumulation with each passing cycle, meaning that additional cycles within the plateau phase are pointless (Figure 5.2).

There are two ways to analyse qPCR data. The first is the relative quantification method, where the amplification of an experimental template is compared to that of a control sample. The relative quantification method is also known as the ΔCt method because one compares the Ct values of unknown samples to the amplification of housekeeping genes or the same gene in a control or reference sample. The purpose of the housekeeping gene is to normalise the PCRs for the amount of RNA added to the reverse transcription reaction. It is therefore important that the expression of internal control or housekeeping genes remains unaffected by the experimental treatment. Figure 5.2 illustrates how the relative expression (2ΔCt) of a gene of interest is calculated relative to a housekeeping gene (normalisation gene). The other method is absolute quantification, which is relatively inaccurate, and used to calculate the actual copy number of template molecules with statistical confidence. It involves comparing the amplification behaviour of an experimental sample against a standard curve and, logically, is sometimes referred to as the standard curve method (Farrell, 2017).

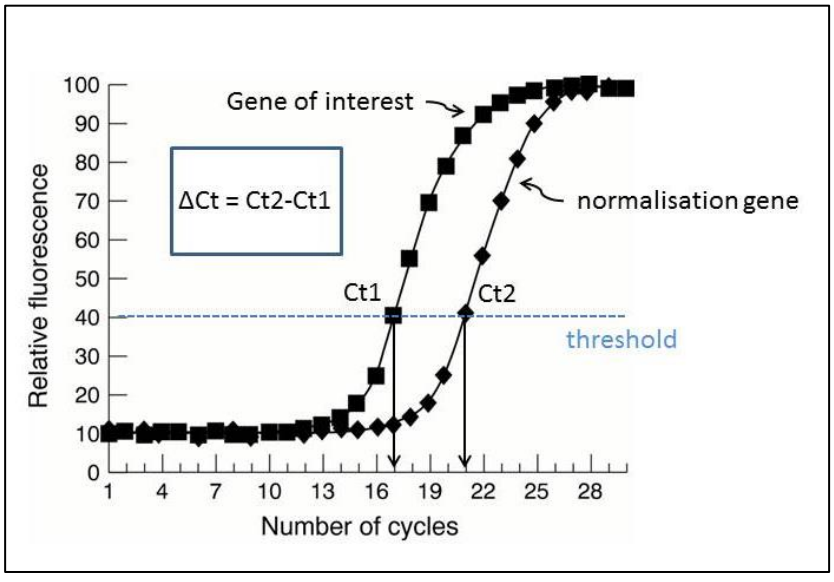

Figure 5.2 Relative expression measure by qPCR.
Relative expression ($2^{\Delta Ct}$) of a gene of interest to a housekeeping gene (normalisation gene). The relative fluorescence of the PCR products increases with an increase number of PCR cycles. The Ct for each gene represents the number of cycles required for the fluorescent signal to cross the threshold. The relative expression of the Gene of interest, $=2^{\Delta Ct}$ (the calculation is done by ΔCt1=Ct2

(housekeeping gene) - Ct1(gene of interest), then calculate the power $2^{\Delta Ct1}$=(power($2^{\Delta Ct1}$) *1000, then calculate the geomean of the two HK genes (GAPDH and RPL4) $2^{\Delta Ct1}$, $2^{\Delta Ct2}$.

5.2.6.2 Extraction of mRNA from harvested liver, lung, gizzard and adipose tissues

Total RNA was extracted using the RNeasy® Mini Kit (50) (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. In brief, tissue samples were first lysed and homogenised in a highly denaturing guanidine-isothiocyanate–containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. The lysate was then passed through a genomic DNA Eliminator spin column. This column, in combination with the optimised high-salt buffer, allows reasonably efficient removal of genomic DNA. Ethanol was then added to the flow-through to provide appropriate binding conditions for RNA, and the sample was then applied to an RNeasy spin column, where total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA was then eluted in 30 µl of water. Total RNA in each sample was quantified using a NanoDrop UV-Vis spectrophotometer (NanoDrop® ND-1000) and ND-1000 V3.1.2 software. After extraction, the total RNA samples were stored at-80^{o}C.

5.2.6.3 Reverse transcription of mRNA to cDNA

Reverse transcription was carried out using the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. In brief, the mRNA samples were diluted with RNAse-free water so that they were at the same final concentration (i.e ~1 µg in a final volume 12 µl). To protect the mRNA from degeneration 2 µl of the supplied Wipeout buffer was then mixed with the diluted samples at room temperature. A master mix was then prepared using 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer and 1 µl RT primer mix and kept on ice. 6µl of the master mix was then added to each sample and the tubes containing the mixture were incubated at 42^{o}C for 30 minutes. To stop the reaction i.e. inactivate the Quantiscript ReverseTranscriptase, the temperature was raised to 95^{o}C for 3 minutes. The total cDNA (ng/ µl) in each sample was then quantified using a NanoDrop UV-Vis spectrophotometer (NanoDrop® ND-1000) and ND-1000 V3.1.2 software. The cDNA samples were then stored at-20^{o}C.

5.2.6.4 Primers

The first 6 primers in Table 5.1, (SAA, AGP and OVT and the 3 housekeeping genes GAPDH, RPL4 and YWHAZ) chosen for this study were selected as reference genes based on previous studies (Marques et al. 2017; Yang et al., 2013; Yue et al., 2010). Additional primers for AGP (AGP-1, AGP-2) and GAPDH (GAPDH-1) were designed using the NCBI tool Primer BLAST platform. The parameters used to design primers were: melting temperature of 63-65^{o}C, with an amplicon length between 160-200bp (Czechowski et al., 2004), and the presence of an intron >1000bp.

Table 5.1 primers sequences.
Sequences of oligonucleotide primers for APP and housekeeping genes (the first 6 primers were obtained from Marques et al., 2017), while the rest were designed as described in the text.

	Gene	Gene bank	Forward primer (5'-3')	Reverse primer (3'-5')	Length (Bp) For., Rev.
1	SAA	XM_003206257.1	TGC TTC GTG TTG CTC TCC AT	CAT GTC CCG GTA TGC TCT CC	20, 20
2	OVT	NM_205304.1	AGC CAT TGC GAA TAA TGA GG	ATG GGC TTC AGCT TGT ATG G	20, 19
3	AGP	NM_204541.2	GGT GTA CAT CAT GGG TGC CT	CGC ATG TTT CAT TCA GCC TCA	20, 21
4	RPL4	XM_003209573.1	TGT TTG CCC CAA CCA AGA CT	TCC TCA ATG, CGG TGA, CCTT T	20, 19
5	YWHAZ	XM_003205203.1	TTC CCT TGC AAA AAC GGC TT	TTC AGC TTC GTC TCC TTG GG	20, 20
6	GAPDH	GQ184819.1	GAT CCC TTC ATC GAC CTG AA	ACA GTG CCC TTG AAG TGT CC	20, 20
7	AGP-1	NM_204541.2	CTC TTC CCC GGC AGCC ATG A	CAC CTG TCC ATC CTC GTG CGT	19, 21
8	AGP-2	NM_204541.2	ATC ACC TCC CCG AAG GAT GCC	GGG CGT CGT GAA GTC CTC GTT	21, 21
9	GAPDH-1	GQ184819.1	TGG GGC CGT TGA CGT GCA G	GCC ACC ACT TGG ACT TTG CCA G	19, 22

5.2.6.5 Real-time quantitative PCR (qPCR)

qPCR was performed using Brilliant II SYBR© Green master mix system (Agilent, Santa Clara, CA US) and the MX PRO MX3000-P software on the computer. The lyophilised primers were first made up to a concentration of 100 pmol/µl by dissolving each in RNase free water according to the instructions provided with each primer. Primer mix was prepared by mixing 20 µl of the forward-primer, with 20 µl of the reverse -primer then adding 60 µl RNase free water to make a final volume of 100 µl. A SYBR green II primer mix solution was then prepared by mixing 23 Brilliant II SYBER green: 1 mixed primer. The amount of this mix prepared depended on the number of wells and plates in each qPCR run.

cDNA samples were diluted 1:60 using LOTE buffer (0.2 M EDTA and 0.5M Tris HCl). 4.8 µl of the SYBR green II primer mix was pipetted in the bottom of each plate well (Thermo Scientific™PCR Plate, 96-well, non-skirted, AB0600) and then 4.8 µl of the diluted cDNA samples was added. Each cDNA sample was tested in duplicate. A typical plate map for n=9 tissue samples using 3 gene primers and 2 house-keeping primers is shown in Figure 5.3. Negative controls were prepared by adding nuclease free water instead of cDNA to the SYBR green II primer mix. These controls were used to make sure no qPCR product was generated from the primers. Positive control samples consisted of using the same 2 cDNA samples (liver) in each plate. The thermal profile used in this study for each primer set was 95˚C for 7.5min, 40 cycles of 95˚C for 25s, 63˚C for 25s and 72˚C for 25s, followed by 1 cycle of 95˚C for 1min, 63˚C for 30s and 95˚C for 30s.

Primer efficiency for all primers listed in Table 5.1 was evaluated using the template dilution method. A five-fold serial dilution of a pooled cDNA sample of liver from the control and treatment groups was prepared and tested using each primer in turn. These were used to create a standard curve. The RSq. value and efficiency level of each primer were assessed in turn. A primer with 100% efficiently expresses double CDNA concentration every thermal cycle.

To check for genomic cDNA, additional samples were prepared using mRNA extracted from 5 liver samples. During the reverse transcription process the enzyme step was omitted from these samples. Thus, in the absence of cDNA no application should take place during the qPCR reaction.

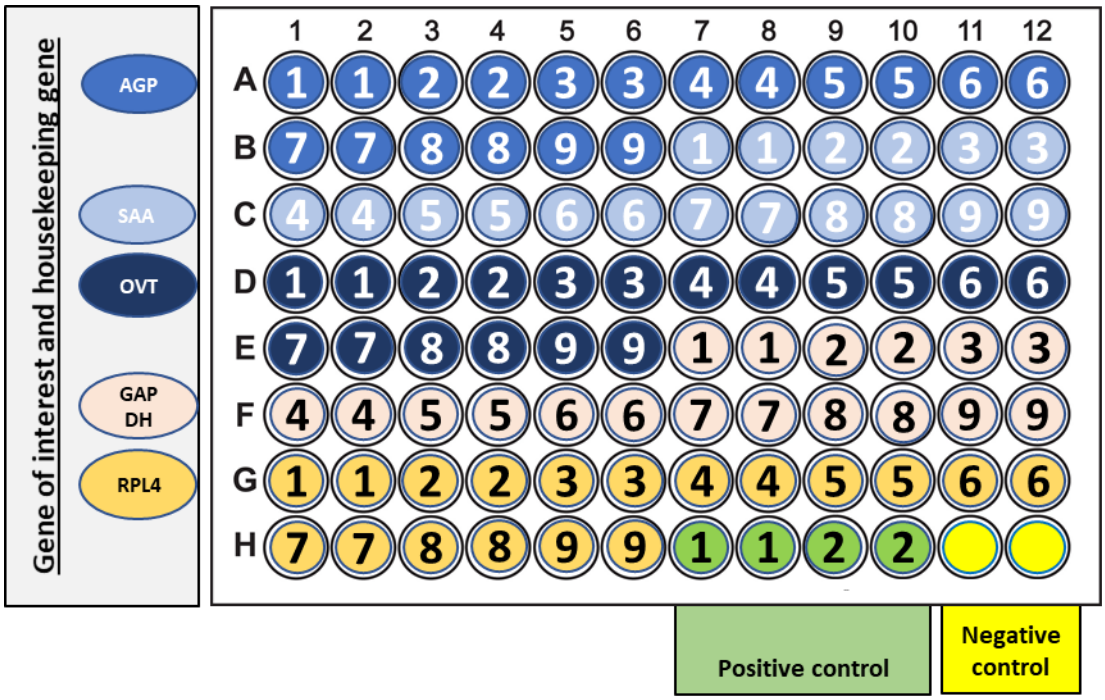

Figure 5.3 Example of layout of a PCR array plate.
Layout of a PCR array plate (Thermo Scientific™PCR Plate). The 96-well, each sample was run in duplicate; each colour represents a primer of 3 genes of interest or 2 housekeeping genes. The green colour well represents the positive control and the yellow wells are the negative control.

5.2.7 Data analysis

Results obtained were initially stored in Excel (Microsoft 2010) and simple descriptive statistics were obtained. Data were later exported to statistical GraphPad Prism software; version v.5 for further analysis.

Tests for normality were run on the acute phase proteins values using normality test . To analyse effects on plasma APP, corticosterone and H /L ratio of LPS challenge in birds. A T-test was used to compare variable beween challaneg and saline group at 0, 6, 12, 24, 48, and 72 h (GraphPad Prism v.5). P-value was considered significant at <0.05.

For qPCR the 2ΔCt for each gene of interest was calculated in Excel sheet by subtracting the number of cycles required for the fluorescent signal to cross the threshold (Ct1) from the Ct2 of each housekeeping gene or normalisation gene. Samples with no Ct values were removed from the data set but only after the RNA extraction and qPCR had been repeated. In the latter case it was assumed that the mRNA had been degraded. For each sample the geomean of the 2ΔCt values for each gene of interest was subsequently computed as recommended by Vandesompele et al., (2002). The use of a geometric mean "normalizes" the ranges being averaged, so that no range dominates the weighting, and a given percentage change in any of the properties has the same effect on the geometric mean. The geometric mean of multiple selected housekeeping genes was validated as an accurate normalization factor (Vandesompele et al., 2002). Grubbs test was then used to identify and remove any outlier’s from the LPS challenge or saline control groups respectively. For statistical analysis the geomean values were then tested for normality and log10 transformed as necessary to avoid heterogeneity of variance. All data were then analysed by one-way ANOVA (Graphpad Prism v.5) using treatment (LPS challenge or Saline control) as the main effect.

5.3 Results

Within 6 h the LPS challenged broiler chickens appeared listlessness, had ruffled feathers, looked depressed, had loss of appetite, and mild diarrhoea with yellowish coloured droppings. The cloacal temperature was also higher in the LPS challenged group than in the saline control group at 6 and 12 h post infection (Figure 5.4). The incremental increase in temperature in addition to the onset of clinical signs indicated that the LPS challenge had been successful. The febrile response in the LPS challenged groups is discussed more fully in the next chapter (section 6.3.1).

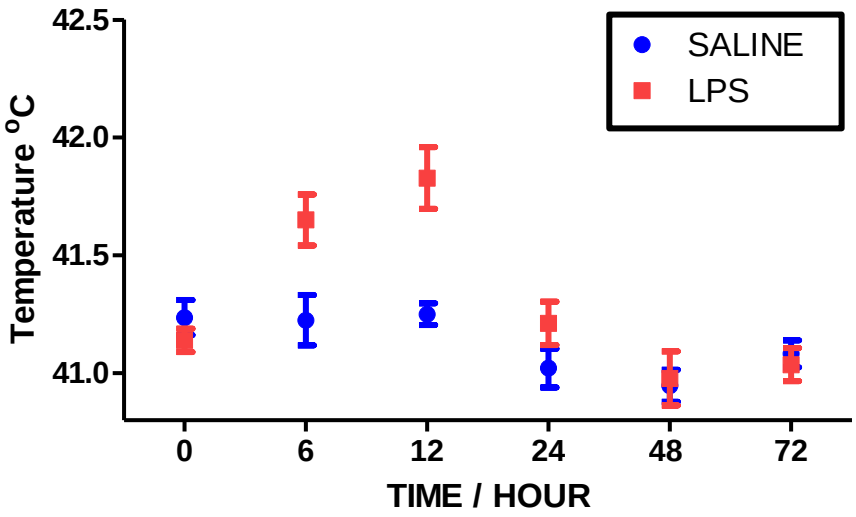

Figure 5.4 Cloacal temperature in response to LPS challenge.
Comparison of cloacal temperature, in saline control (blue) (n=24 per time point) and LPS challenged birds(red) (n=24 per time point), over the time course of this study. (***=P< 0.001). Data presented as mean with SEM shown as the error bar.

5.3.1 Blood parameters

At 6 and 12 h post treatment it was not possible to collect blood sample all of the birds in each replicate. This was because of difficulties experienced in the initial blood sampling which caused hematomas to develop in some individuals. The following blood results were therefore generated from different numbers of individuals at each time point. At 6 and 12 h at least 12 individuals were sampled but they were not necessarily the same individuals. From 24 h to the end of the experiment most but not all individuals per group were sampled. The data set

described below should therefore be interpreted with this in mind. The number of individual samples are indicated in the accompanying figures.

5.3.2 Heterophil / Lymphocyte (H /L) ratio

At 24 h the H/L ratio was 4.4-fold higher in the LPS challenged group than the control saline group. The H/L ratio was also 3-fold higher in the LPS group at 48h (Figure 5.5a; P<0.001). There was no statistical difference between the groups at 72 h (Figure 5.5C; P=0.634)

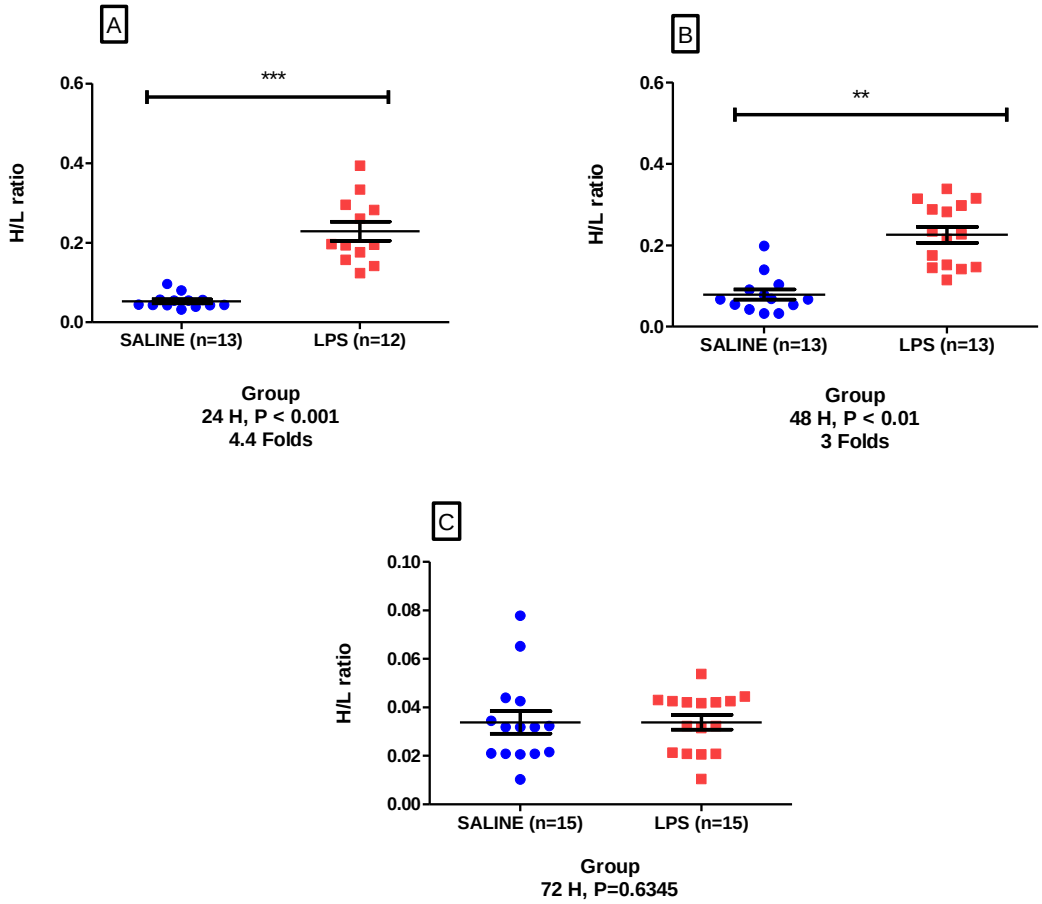

Figure 5.5 Influence of LPS or saline treatment on the H/L ratio.
Scatter plot comparing Heterophil and Lymphocyte (H/L) ratios in LPS challenged and saline control groups at 24, 48 and 72 h post treatment. Mean (horizontal bar), ± SEM and level of significance is indicated *** = P ≤0.001; ** P<0.01.

5.3.3 Corticosterone levels

Table 5.2 compares the mean and SEM concentration of corticosterone in the same 12 LPS challenged and saline control birds at 0 and 6, 12, 24, 48 and 72 h post treatment. A significant difference ($P<0.001$) was observed at 6 h post treatment. The corticosterone level at 6 h post treatment was 3.5-fold higher in the LPS challenged birds (Figure 5.6). No other significant differences were observed.

Table 5.2 Corticosterone concentration in response to LPS challenge.
Comparison of corticosterone concentration at each time point. The data present as the mean ± SEM of the concentration of the corticosterone in saline and LPS groups. the P value shows no significant difference in all time points except at 6 h.

Time Hour	Saline Mean ± SEM(n=12)	LPS Mean ± SEM (n=12)	P value
0	13.15 ± 1.29	9.12 ± 0.861	0.093
6	7.36 ± 0.895	28.65 ± 0.892	0.003
12	10.49 ± 1.597	10..26 ± 1.075	1.000
24	10.17 ± 1.506	9.56 ± 0.823	0.792
48	10.39 ± 0.791	7.68 ± 0.954	0.318
72	12.28 ± 0.419	12.92 ± 1.253	0.636

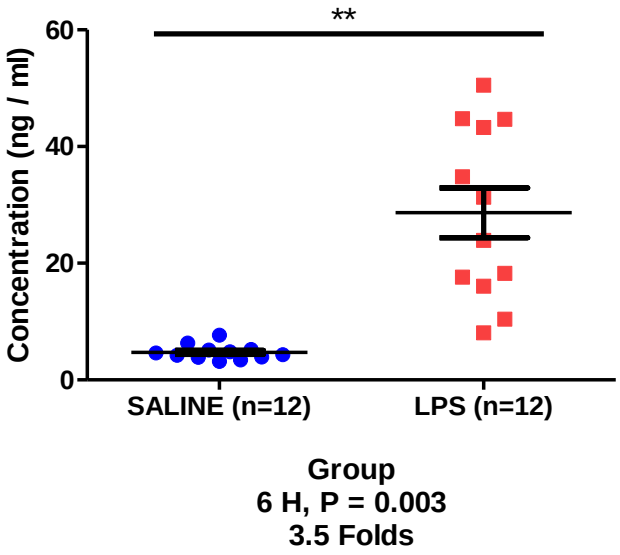

Figure 5.6 Corticosterone concentration at 6 h post treatment.
Scatter plot of corticosterone concentration in saline (n=12) and LPS (n=12) birds. Mean (horizontal bar), ± SEM and significance is indicated ** = P ≤0.01.

5.3.4 Acute Phase Proteins

5.3.4.1 Plasma concentrations of SAA

The mean and SEM concentration of SAA in the LPS challenged and saline control groups at each time point is illustrated in Figure 5.7. SAA levels in the LPS challenged group peaked between 6 and 12 h post treatment then decreased again. SAA levels in the control group were consistently low throughout the experiment.

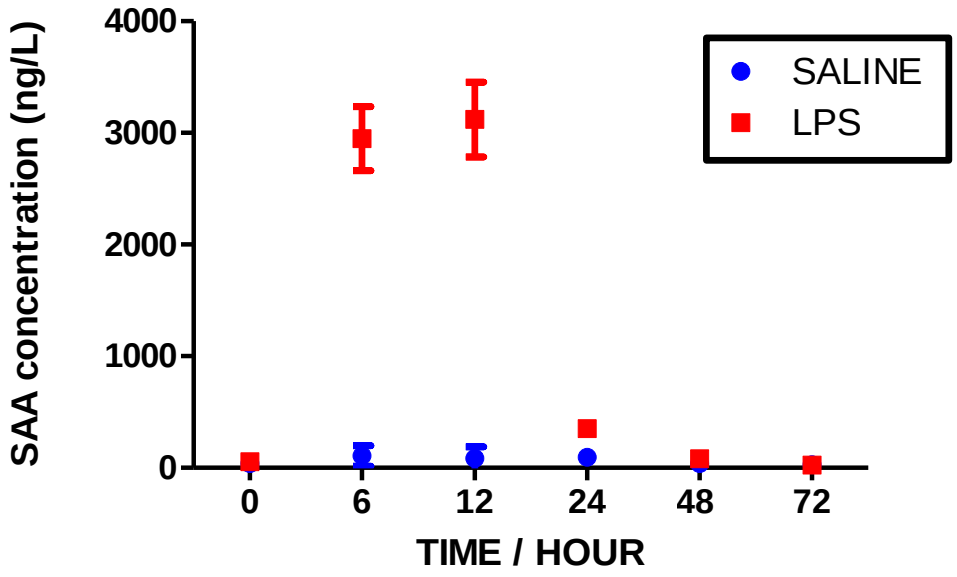

Figure 5.7 SAA response pattern to LPS challenge.
SAA concentrations in LPS challenged and saline control groups across the time course of the study. Data presented as mean and SEM. Note that not all birds were sampled at each time point. The number of samples for each group at each time point and statistical analysis is detailed in figure 5.8.

Figure 5.8 compares the data from each treatment at each individual time point and indicates where statistical differences were observed. At 6 h post treatment the SAA levels in the LPS challenged group were 27 fold higher ($P<0.001$) than in the control group and at 12 h they were 32 fold higher ($P<0.001$). The SAA levels in the LPS challenged birds remained significantly higher than the control birds at both 24 and 48 h although in absolute terms the difference between the two groups was much smaller (between 2-3 fold of a difference) than previously observed. By 72 h there were no differences observed between the two treatments.

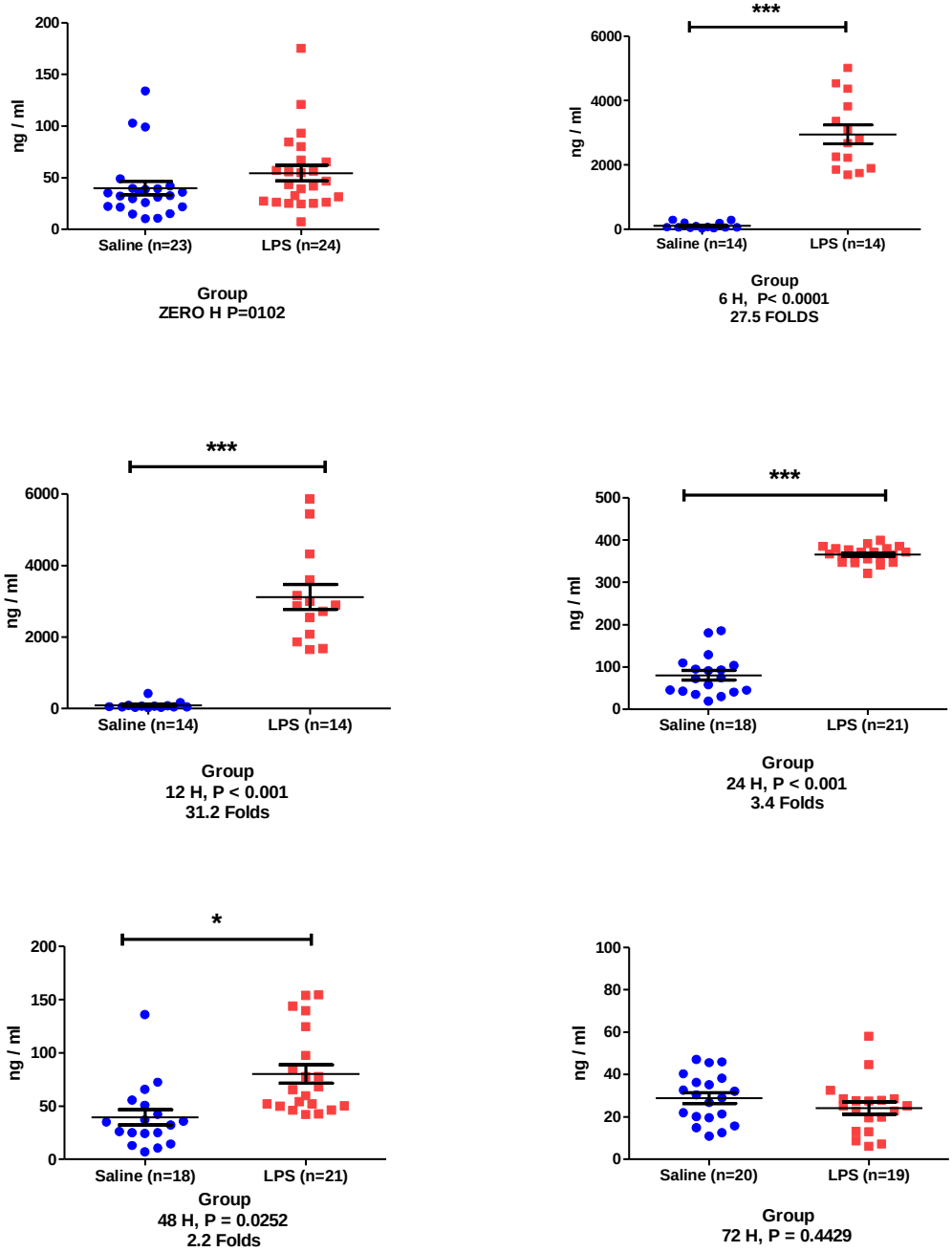

Figure 5.8 SAA levels at each sampling time point following LPS challenge.
Scatter plot of SAA concentrations of saline control and LPS groups at 0, 6, 12, 24, 48 and 72 h post treatment with mean (horizontal bar) and ± SED. (***P<0.001; ** P<0.01; *P<0.05). Note the change in magnitude of scale on the y-axis in the plots for 6 and 12 hs.

5.3.4.2 Plasma concentrations of AGP

The mean and SEM concentration of AGP in the LPS challenged and saline control groups at each time point is illustrated in Figure 5.9. AGP levels in the LPS

challenged group peaked at 12 h post treatment then gradually declined over the time course of the study. AGP levels in the saline control group were consistently low although there was a slight increase then a decrease observed.

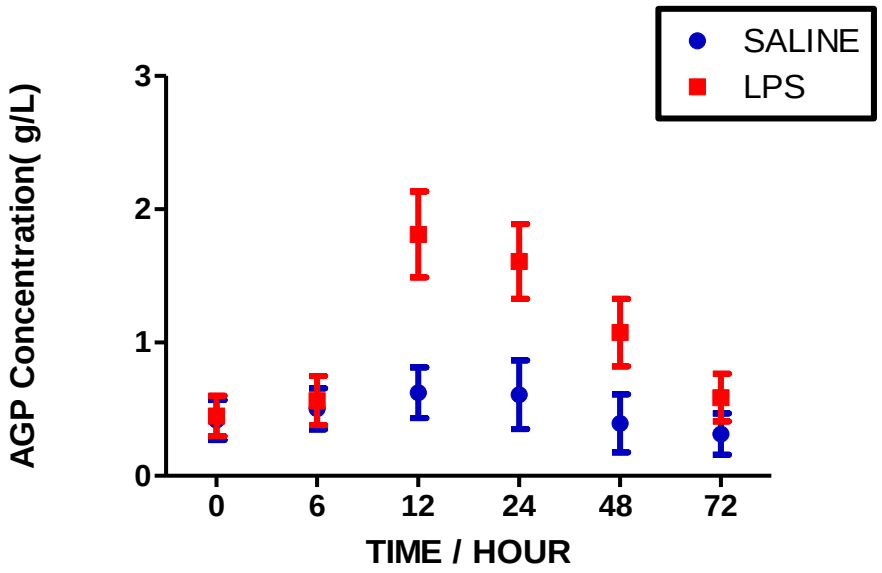

Figure 5.9 AGP response pattern to LPS challenge.
AGP concentrations in LPS challenged and saline control groups across the time course of the study. Data presented as Mean and SED. Note that not all birds were sampled at each time point. Number of samples for each group at each time point and statistical analysis is detailed in figure 5.10.

Figure 5.10 compares the AGP data from each treatment at each individual time point and indicates where statistical differences were observed. At 12 and 24 h post treatment AGP levels in the LPS challenged group were 3-fold higher ($P<0.001$ in each case) in the LPS challenged group than the saline control group, at 48 h they were 2.5-fold higher ($P<0.001$) and at 72 h they were still 1.8-fold higher($P<0.01$).

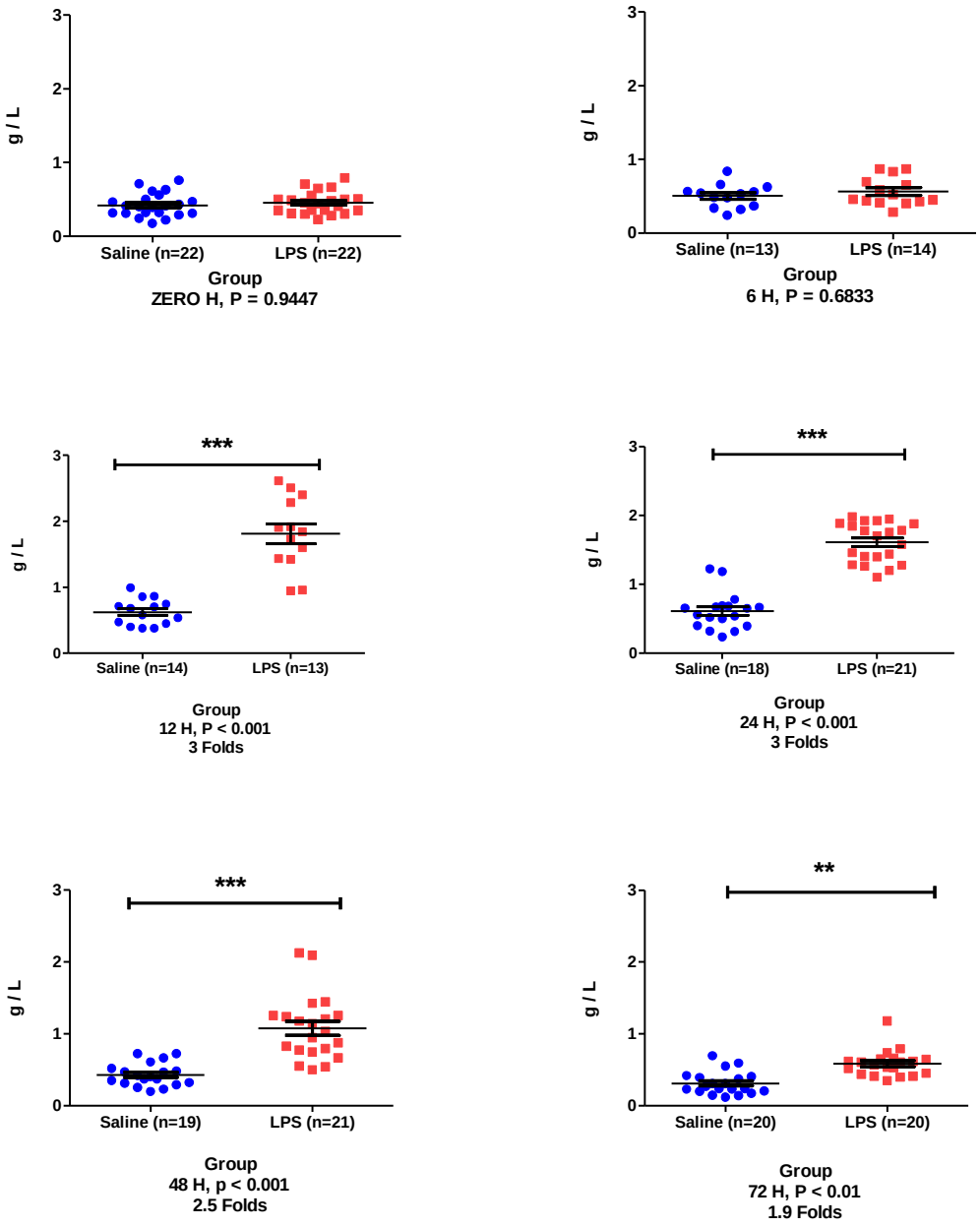

Figure 5.10 AGP levels at each sampling time point following LPS challenge.
Scatter plot of AGP concentrations of saline control and LPS challenged groups at 0, 6, 12, 24, 48 and 72 h post treatment with mean (horizontal bar) and ± SEM. (***P<0.001; ** P<0.01; *P<0.05).

5.3.4.3 Plasma concentrations of OVT

The mean and SEM concentration of OVT in the LPS challenged and saline control groups at each time point is illustrated in Figure 5.11. OVT levels in the LPS challenged group peaked at 24 h post treatment then gradually declined over the time course of the study. OVT levels in the saline control group were consistently low throughout the experiment.

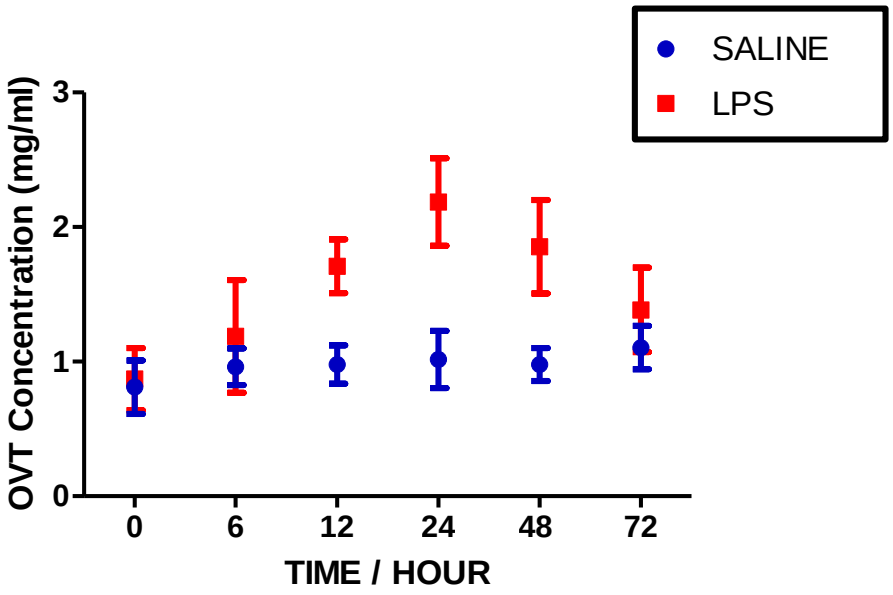

Figure 5.11 OVT response pattern to LPS challenge.
OVT concentrations in LPS challenged and saline control groups across the time course of the study. Data presented as mean and SEM. Note that not all birds were sampled at each time point. Number of samples for each group at each time point and statistical analysis is detailed in figure 5.12.

Figure 5.12 compares the OVT data from each treatment at each individual time point and indicates where statistical differences were observed. Significant differences in OVT were observed at all sampling time points except time 0. At 6 h post treatment a 1.3 fold difference ($P=0.02$) was observed between the LPS challenged and saline control groups. At 12 h the difference increased to 1.7 fold ($P<0.001$) and by 24 and 48 h there was a 2 fold difference between the two groups ($P<0.001$ in each case). By 72 h the difference between the two groups had decreased to 1.3 but this difference was still significant ($P=0.01$).

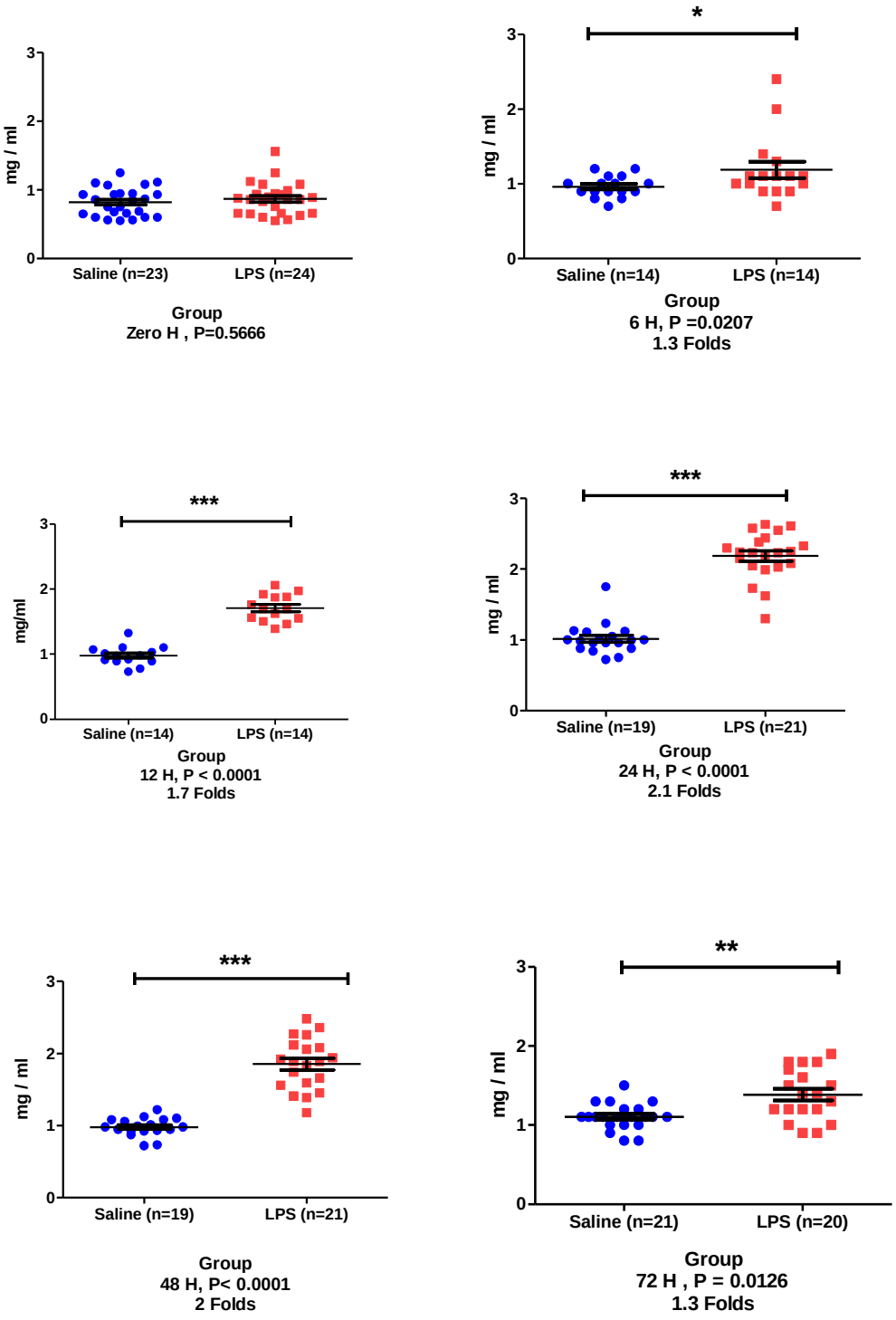

Figure 5.12 OVT levels at each sampling time point following LPS challenge.
Scatter plot of OVT plasma concentrations of Saline and LPS groups birds at 0, 6, 12, 24, 48 and 72 h post treatment with mean (horazontal bar) and ± SEM (***P<0.001; ** P<0.01; *P<0.05).

5.3.5 Expression of *AGP*, *SAA* and *OVT* mRNA in the liver, and selected extrahepatic tissues

5.3.5.1 Primer efficiency test

In a preliminary set of experiments, the primer quality was assessed by qPCR (Figure 5.13). The acceptable RSq. value was taken as >0.9 and the efficiency as >80%. GAPDH was not used because it had low efficiency (Table 5.3). AGP was not used because the results of each sample within the group were not harmonised and hugely differed from each other. Therefore, new GAPDH and AGP primers (GAPDH-1, AGP-1 and AGP-2) were designed and, after repetition of the experiments, the results were better for AGP-1 and GAPDH-1 (Table 5.3). The YWHAZ housekeeping gene was not used because the RSq. value and efficiency level of the primers did not generate meaningful data in the efficiency tests. Also, AGP-2 was not used because of low efficiency level. Hence, the finalised primers and housekeeping genes selected for the study were SAA, OVT, AGP-1 (new), GAPDH-1 (new) and RPL4.

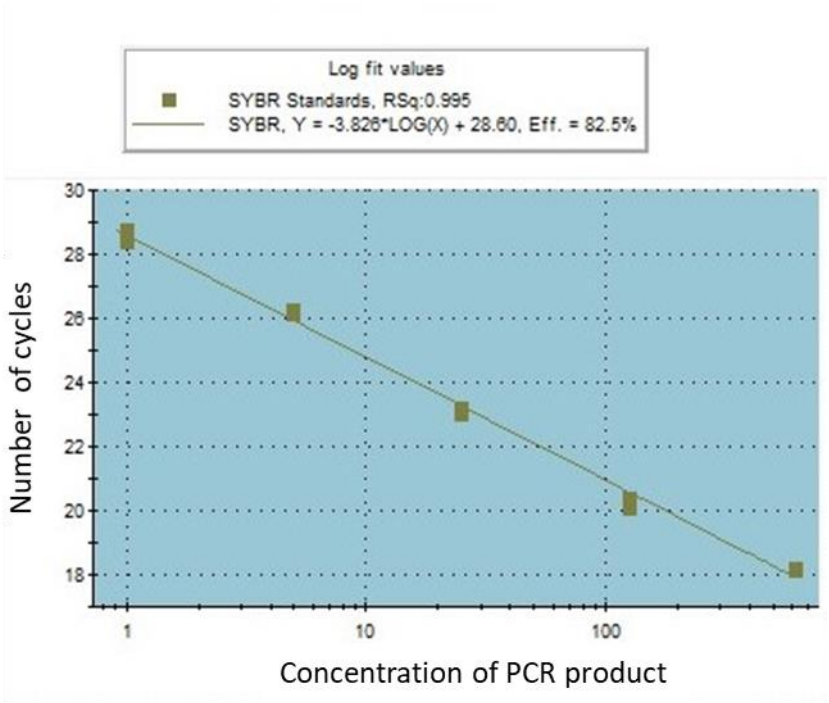

Figure 5.13 An example of the primer efficiently test (AGP-1 primer).
The line graph shows a serial dilution of the pooled sample which gave efficiently higher than 80% and R more than 0.9.

Table 5.3 Primer efficiency tests:
Correlation coefficients (RSq) values and efficiency level % for all primers. Those in red were selected for use in this study.

	Primer	RSq.	Efficiency level (%)
1	AGP	0.967	138.9
2	SAA	0.983	139.6
3	OVT	0.987	119.0
4	GAPDH	0.945	77.3
5	RPL4	0.914	161.4
6	YWHAZ	0.001	>1000
7	GAPDH-1	0.983	82.9
8	AGP-1	0.995	82.5
9	AGP-2	0.973	76.0

5.3.5.2 *SAA, AGP* and *OVT* mRNA expression levels in the Liver of LPS challenged and saline control birds at 72h PT.

The mRNA expression levels of *SAA, AGP* and *OVT* in the liver of LPS challenged birds and the saline control birds are compared in Figure 5.14 A-C. The mRNA expression levels of *OVT* were significantly higher (2.3-fold increase) in the liver of the LPS challenge group compared to the saline controls (Figure 5.14C (P=0.05). There was no difference between the groups in terms of the liver mRNA expression levels of *AGP* and *SAA* (Figure 5.14A-B).

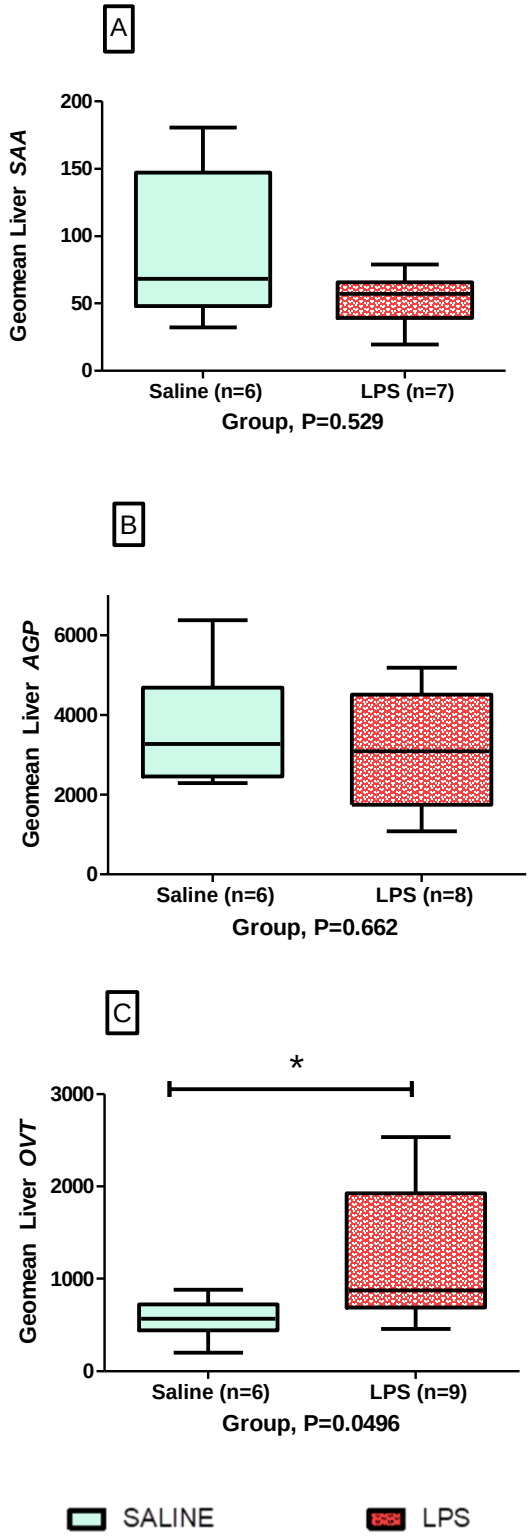

Figure 5.14 APP expression in liver samples of chickens challenged with LPS.
Comparison of *SAA, AGP* and *OVT* mRNA expression levels in the liver of LPS challenged and saline control birds at 72 h P.T. *OVT* expression was significantly higher in LPS challenged group compared to the saline control group. Data presented as box and whisker plots with median in the box and 25-75 percentile ranges as the box and the whisker as 10-90percentiles. *P=0.05).

5.3.5.3 *SAA, AGP* and *OVT* mRNA expression levels in the Lung of LPS challenged and saline control birds at 72h P.T.

The mRNA expression levels of *SAA, AGP* and *OVT* in the lung of LPS challenged birds and the saline control birds are compared in Figure 5.15 (A-C). No significant differences were observed.

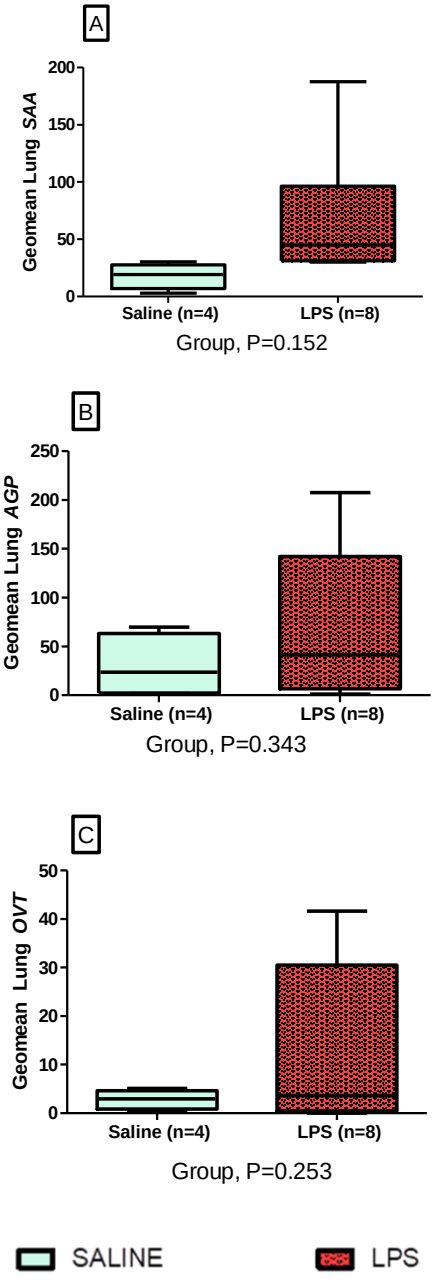

Figure 5.15 APP expression in lung samples of chickens challenged with LPS.
Comparison of *SAA, AGP* and *OVT* mRNA expression levels in the lung of LPS challenged and saline control birds at 72 h P.T., data presented as box and whisker plots with median in the box and 25-75percentile ranges as the box and the whisker as 10-90 percentiles)

5.3.5.4 *SAA, AGP* and *OVT* mRNA expression levels in the gizzard of LPS challenged and saline control birds at 72h P.T

The mRNA expression levels of *SAA, AGP* and *OVT* in the gizzard of LPS challenged birds and the saline control birds are compared in

Figure 5.16 (A-C). The *OVT* mRNA expression levels were significantly higher in the LPS challenged group (

Figure 5.16 C, P=0.03). No other statistically significant differences were observed in the gizzard.

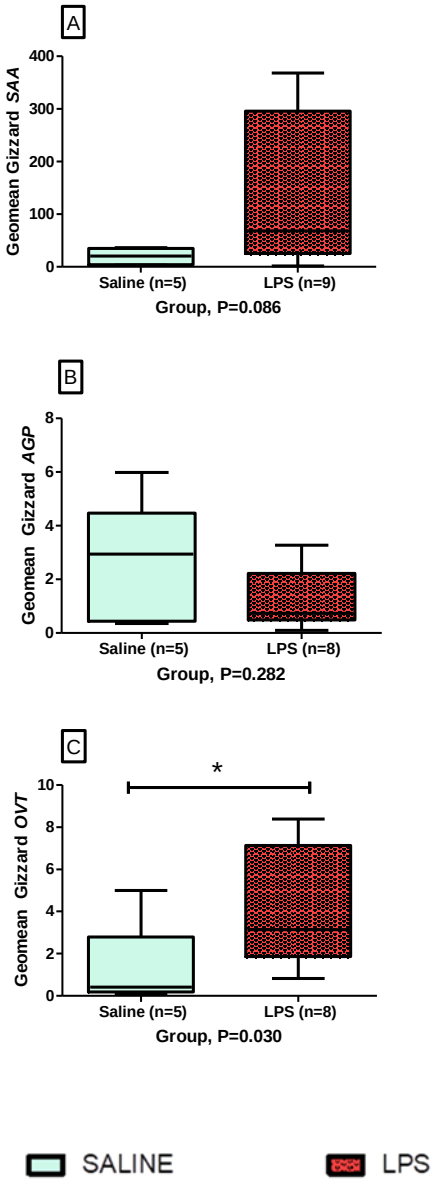

Figure 5.16 APP expression in gizzard samples of chickens challenged with LPS.
Comparison of *SAA, AGP* and *OVT* mRNA expression levels in the gizzard of LPS challenged and saline control birds at 72 h P.T., data presented as box and whisker plots with median in the box and 25-75percentile ranges as the box and the whisker as 10-90percentiles. *P=0.05).

5.3.5.5 *SAA, AGP* and *OVT* mRNA expression levels in adipose tissue of LPS challenged and saline control birds at 72 h P.T.

The mRNA expression of *SAA* and *OVT* in adipose tissue was consistently low in both the LPS challenged group and the saline controls (Figure 5.17 A-B). Indeed, there was no detectable expression of *AGP* in either group (data not shown).

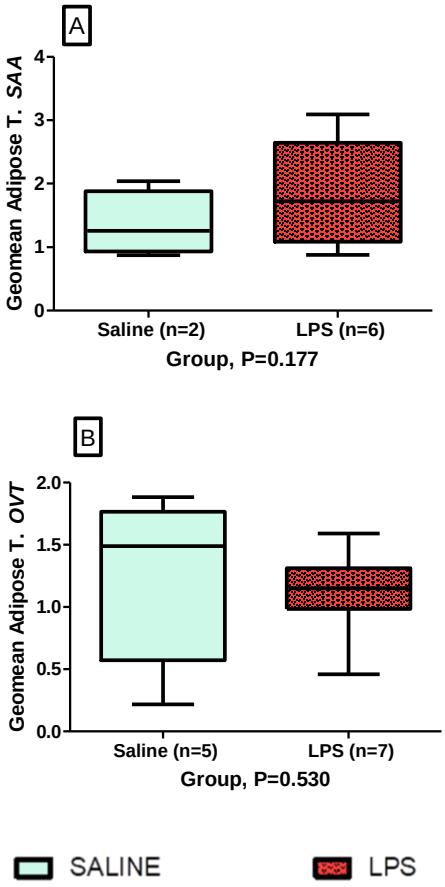

Figure 5.17 APP expression in adipose tissue samples of chickens challenged with LPS.
Comparison of SAA and OVT mRNA expression levels in adipose tissue of LPS challenged and saline control birds at 72 h PTI. Data presented as box and whisker plots with median in the box and 25-75percentile ranges as the box and the whisker as 10-90percentiles).

5.3.5.6 Extrahepatic mRNA expression relative to the liver

Extrahepatic mRNA expression levels of *SAA, AGP* and *OVT* relative to the liver (=1) in LPS challenged and saline control birds are presented in

Figure 5.18 (A-C). Overall, the extrahepatic mRNA expression of *AGP* and *OVT* was very low compared to that in the liver. In contrast, the mRNA expression levels of *SAA* in the LPS challenged birds was higher in the lung and gizzard than in the liver at 72 h P T (Figure 5. 18 A).

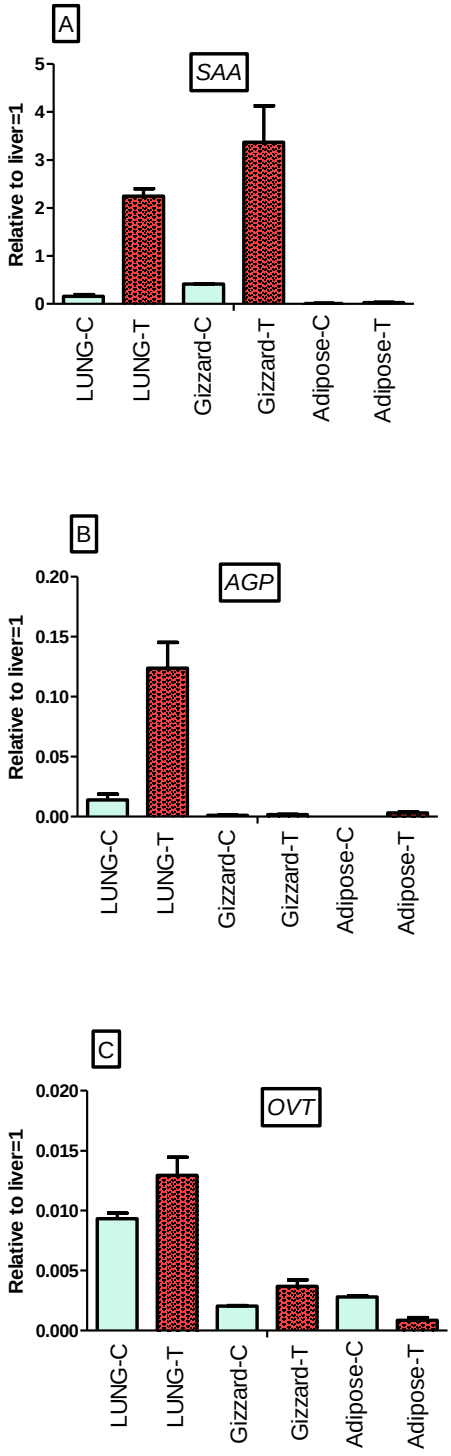

Figure 5.18 Relative extrahepatic expression of APP in chickens challenged with LPS. Extrahepatic tissue expression of (A) *SAA* mRNA, (B) *AGP* mRNA and (C) *OVT* mRNA in LPS challenged and saline control birds relative to that in the liver (=1) at 72h P T Data shown as mean ±SEM. Red bars with dots are LPS group and the light green are the saline group.

5.4 Discussion

The first aim of this chapter was to measure and compare the plasma levels of corticosterone, the H/L ratio and plasma levels of three APP (AGP, SAA and OVT) in broiler chickens following an LPS challenge over a time course of 72 h (six times sampling). During the early stages of the experiments (time 0) unforeseen problems were encountered which meant that it was not always possible to then sample blood from the same individuals birds at 6 and 12 h. Nevertheless, the results show that at 6 h post challenge, the plasma levels of corticosterone and SAA were significantly increased in the LPS challenged group. This time point also happens to coincide with the time at which the LPS challenged birds started to display a febrile response. At 12 h post challenge there was no difference in corticosterone levels but the plasma levels of all 3 APP were significantly higher in the LPS challenged group. The plasma levels of AGP and OVT remained higher in the LPS challenged birds until the end of the experiment whereas the SAA levels were similar to those of the saline controls at 72 h. Unfortunately, there is no data for H/L ratios prior to 24 h post challenge but at both 24 and 48 h the ratios were higher in the LPS challenged group.

Glucocorticoids increase during the APR as a result of cytokine-stimulation of the pituitary-adrenal axis, which increases secretion of adrenocorticotrophic hormone (Klasing and Johnstone, 1991). Thus, an increase of corticosterone will occur later than the appearance of IL-6 (Amrani et al., 1986). The observed elevation in corticosterone concentration at 6 h post challenge is therefore consistent with the production of pro-inflammatory cytokines following LPS administration. Corticosterone acts to balance cytokine production (Figure 1.1) by down regulating pro-inflammatory cytokines, like IL-1, IL-16, TNF-α, and interferon-γ, while upregulating anti-inflammatory cytokines such as IL-10, IL-4, and TGF-ß (Sapolsky et al., 2000; Elenkov, 2004). The balance between the pro and anti-inflammatory cytokines will determine the final APP response of the animal to stressors (Shini et al., 2008). Corticosterone release essentially protects the animal from a systemic over-activation of inflammatory cytokines (Sapolsky et al., 2000) and is therefore an essential part of the stress related response to foreign antigens (Siegel, 1985, 1995; Khansari et al., 1990).

Acute-stress related increases in glucocorticoids are known to affect multiple physiological body systems as well as the number of circulating white blood cells. In birds, for example heterophils increase as a result of migration from the bone marrow and/or the marinated pool into the blood stream (Post et al., 2003; Shini et al., 2008). The observed increase in H/L ratio in the LPS challenge group at 24 and 48 h is consistent with this finding, and with the results previously reportedin 7 day old chickens vaccinated with Newcastle and Infectious bronchitis vaccine in this thesis .

As previously stated there was a significant increase in all 3 APP in the LPS challenged group but the timing and magnitude of the difference observed was protein specific. SAA significantly increased in the LPS challenged group by 27 and 32-fold after 6h and 12 h post treatment. Furthermore, the level of change in SAA measured in this experiment was much greater than that observed in the vaccine trial (2.5 fold) as discussed in Chapter 3. These results confirm that SAA is a major APP protein in chickens (O'Reilly and Eckersall, 2014).

For AGP a 3-fold significant difference was observed between the LPS challenged and saline control groups at 12h. For OVT a 1.3-fold significance difference was observed at 6 h which increased to a 2-fold difference at 24 h. These results are consistent with other LPS-based studies (Koppenol et al., 2015, Packialakshmi et al., 2016). However, these last-mentioned studies did not measure the SAA concentration and they also did not follow the time course of the APP response for AGP or OVT. The current study has shown that both AGP and OVT remained significantly higher in the LPS challenged birds for up to 72 h post challenge.

The second aim of this chapter was to investigate the mRNA expression levels of SAA, AGP and OVT in the liver and a range of extrahepatic tissues in both the control and challenged birds by qPCR. This is the first study on broiler chickens that has adopted this approach. mRNA for AGP, SAA and OVT was detected in the liver but also in the lung and gizzard tissues sampled from both the LPS and saline control groups at 72 h. SAA and OVT mRNA was also detected in the adipose tissue. These results confirm that extrahepatic tissues are capable of APP synthesis both in normal and under challenge conditions. As anticipated there was no difference between groups in the SAA expression level in the liver or any of the extrahepatic tissues at 72 h. This is consistent with the finding that the plasma levels of SAA

which had been previously elevated in the LPS group had returned to normal by 72 h post challenge. The fact that the mRNA expression levels for SAA remained higher in the lung and gizzard compared to the liver in the LPS challenge group suggests that the local response to the stimulus was slower to recover. As previously stated, AGP and OVT plasma levels were still elevated in the LPS control group at 72 h. For OVT this finding is consistent with the finding that there were differences in mRNA OVT expression levels observed in both the liver and gizzard tissues of LPS challenged birds. This finding suggests that these extrahepatic tissues could be contributing to the circulating plasma levels of OVT during the recovery period. For AGP, mRNA AGP expression levels did not differ in the liver nor any of the extrahepatic tissues tested. In this case it could be that extrahepatic tissues other than those tested here were contributing to the circulating levels of AGP.

A major limitation of this study is that funding was only available to study the mRNA expression of SAA, AGP and OVT in the liver and 3 extrahepatic tissues. A further limitation was that it was only possible to harvest tissues at the end of the experiment (72 h). Ideally a subset of birds from both the LPS and control groups would have been euthanized at 0, 6, 12, 24, 48 h but for practical reasons this was not possible.

Despite these limitations, studies in other species support the main findings of this study (Vernooy et al., 2005). In mice an intratracheal LPS challenge between 8 to 10 weeks resulted in a significant elevation in mRNA expression of APP in pulmonary tissues at 4, 24 and 72 h post treatment. In another study involving 8 to 10 weeks old pigs inoculated intranasally with *A. pleuropneumoniae*, APP gene expression was significantly elevated many extrahepatic tissues 14 h post treatment (Skovgaard, 2009). The findings of the current work therefore add to existing evidence in other species namely that local extrahepatic mechanisms contribute to the regulation of the immune response.

In conclusion, subcutaneous injection of LPS in broiler chickens, induced the onset of an endotoxemia, with clinical signs, after 6 h. LPS triggered APR causing an elevation SAA, OVT and AGP in the blood plasma. The SAA increases were detected first and were greatest magnitude, therefore this is the most sensitive APP. Furthermore, corticosterone and H/L ratio increased significantly at 6 h and 1-2

days respectively. At 72 h PT the systematic concentrations of AGP and OVT were still elevated in the LPS group.

Generally, the expression of SAA, and AGP in the liver and the lung, gizzard and adipose tissue were comparable in the control and challenged group. The exception was OVT which was expressed more in the liver and gizzard of the LPS challenged group. Overall the extrahepatic expression of APP was low in both groups; this could be due to the late collection of samples.

Chapter 6 Infra-red thermography as a non-invasive method of monitoring body surface temperature changes associated with pyrexia in broiler chickens

6.1 Introduction

In commercial poultry production the early detection of disease is essential in implementing effective therapy, preventing economic loss and protecting bird welfare. However, traditional clinical or serological examination of large numbers of birds is logistically and economically challenging. Infra-red thermography (IRT) has been used in humans and animals as a non-invasive method to estimate physiological or pathological changes in body surface temperature resulting from the administration of pharmaceuticals, surgical procedures, changes in blood flow or local inflammatory responses to disease conditions (Clark, 1977; Schaefer et al. 1988; Spire et al. 1999; Cockcroft et al., 2000; Scott et al., 2000; Eddy et al., 2001; Heath et al., 2001). More recently, IRT has been used to monitor respiratory disease (Schaefer et al., 2007) and viral diarrhoea in calves (Schaefer et al., 2004), back pain in horses (Fonseca et al., 2006), hoof disorders of cows (Nikkhah et al., 2005), and the effect of milking on bovine teat tissue (Paulrud et al., 2005). These studies show that IRT can assist in the identification of a health issues in a range of animal species and could promote the early detection and diagnosis of a range of clinical conditions.

In avian biology, IRT has been used successfully to detect changes in body surface temperature for decades (McCafferty, 2013). Examples include studying heat loss and air temperature in Arctic birds (Veghte and Herreid, 1965), heat loss and thermoregulation in the ostrich (Phillips and Sanborn, 1994), feather cover score in domestic fowl (Cook et al., 2006), hen egg temperature during incubation and exposure to the cold (Shinder et al., 2009), the correlation between facial and cloacal core temperature in hens exposed to different environmental conditions (Giloh et al., 2012), and as a useful tool for the early detection of subclinical leg pathologies (bumble foot) in laying hens (Wilcox et al., 2009).

IRT has also been shown to be a useful tool for measuring the body surface temperature changes that are associated with stress in birds (Edgar et al., 2011 and 2013), including identifying the intensity of acute stress (Herborn et al., 2015). Acute stressors induce cutaneous vasoconstriction, leading to a rapid drop in skin temperature (Herborn et al., 2015, Edgar et al., 2013) and a rise in body core temperature, termed stress induced hyperthermia (SIH) (Cabanac and Aizawa, 2000). SIH is followed by a subsequent vasodilatation in order to dissipate

excess heat resulting in a post-stressor rise in peripheral temperature (Schaefer et al., 2004). These changes are transient and thus distinguishable from the sustained elevation in body surface temperature that is associated with pyrexia (Hovinen, et al., 2008). Pyrexia is a common symptom of many pathological conditions (Anochie, 2013) and is the result of a very complex process involving cytokines that act upon the thermoregulatory centre in the hypothalamus to reset the normal temperature level (Dinarello, 2004). As a consequence, considerable excess heat has to be radiated from the body surface and much of this (40–60%) is expressed in the infra-red range (Kleiber, 1967).

The commonest way of recording the body core temperature of a chicken is to place a probe about 1cm into the cloaca which is invasive, time consuming and potentially could result in tissue trauma. The aim of this study was to establish if IRT can be used as a non-invasive method to detect the early onset of pyrexia in broiler chickens, using the model of an LPS challenge. The objectives were to a) identify body surface temperature changes associated with pyrexia using IRT and, b) establish the relationship between body surface temperature changes in different regions and core (cloacal) body temperature in both normal and pyretic individuals. With localised inflammation, for example, you would not expect core and surface temperatures to correlate, whereas during pyrexia we would expect this correlation since surface temperature will increase to promote dissipation of excess heat following the change to the hypothalamic set point.

6.2 Materials and methods

6.2.1 Birds, Housing and Treatment

This study was carried out using the same birds and in parallel to the *E. coli* endotoxin (LPS) challenge experiment presented and described in chapter 5. (Section 5.2). In brief, the experiment was set up such that there were 12 birds in 4 pens. At 15 days of age all birds in pens 1 and 3 were given a subcutaneous injection (SC) of *E. coli* lipopolysaccharide (LPS, dose 2mg/ml) and the birds in pens 2 and 4 were given a SC injection of sterile saline (equivalent volume) as a control for the effects of handling and injection. The study was conducted under Home Office Authority under Project and Personal Licences.

6.2.2 IRT image capture

A single thermal image of each bird was captured at 0 h (pre-treatment) and at 3, 6, 9, 12, 24, 48 and 72 h post treatment (LPS challenge or saline control) using a FLIR SC640™camera with the following specifications: sensitivity <0.1ºC, accuracy ±2. Each bird was gently removed from the pen and placed in a defined area directly in front of the camera. This ensured a fixed 90cm distance between the bird and the camera lens in each image. Each bird was then photographed from the side facing to the right (Figure 6.2), with the head and body position in as similar a position as possible each time as this can affect the temperature estimation (Herborn et al., 2015). The ambient room temperature and humidity were recorded (portable Thermo-Hygrometer PTH-338) during each image capture session (temperature range: 23.2-25.8°C; Relative humidity range: 36-41%).

6.2.3 IRT image processing, regions of interest (ROI) and body surface temperature measurements

Thermal images were processed following the method described by Herborn et al. (2015). In brief, the raw images were processed using FLIR Thermacam Researcher Pro 2.10™ as shown in Figure 6.1.

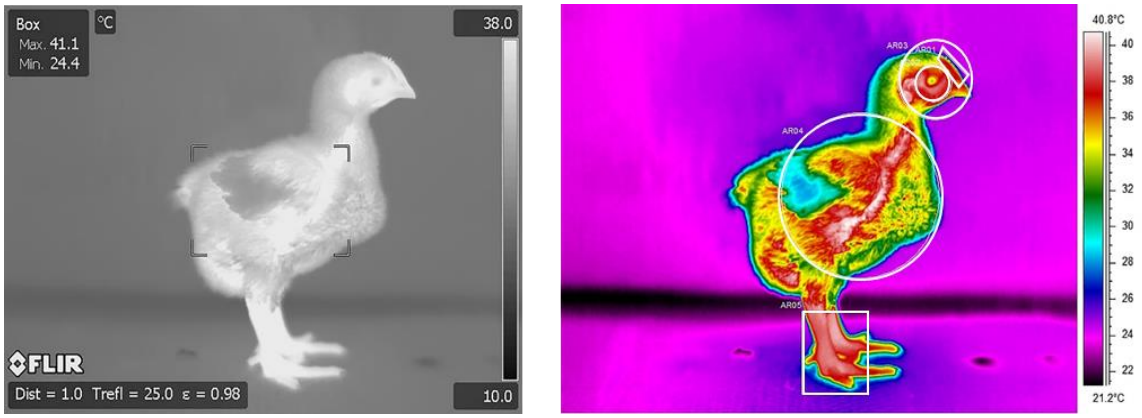

Figure 6.1 Example IRT images of a broiler chicken.
The left image (raw image) shows the hottest areas in white, the coolest areas as black, according to the grey scale(10-38oC) indicated to right of the image. The right image has been false coloured, and the span optimised with FLIR ResearchIR software. The range in temperature is indicated according to the scale on the right which ranges from 21.2-40.8 oC with red representing the hottest regions and magenta the coolest. Five regions of interest (ROIs) are indicated on the colour image (head, eye, comb, wing area and legs). The maximum temperature in each ROI along with that for the whole image was derived for each false coloured image.

The free-hand drawing tool was then used to define five regions of interest (ROI) on each image, namely the eye region, comb, head, wing area and legs. The highest surface temperature in each ROI along with the highest surface temperature for the whole image (image-max) were then extracted by virtue of the software from the data set after setting the emission to 0.97 and adjusting for any changes in the ambient room temperature and relative humidity for each image capture session. A >95% within-observer repeatability in demarcation of the ROIs was established as previously reported by (Herborn et al., 2015).

6.2.4 Cloacal temperature measurements

This was measured as described in chapter 5 (section 5.2.1) but only after the IRT image capture of each bird had been completed (so as not to confound surface temperature measurements with an acute stress response). Cloacal temperature measurements were not carried out at 6h and 9 h post treatment to reduce the stress of repeated cloacal probe penetration and minimize any risk of tissue trauma.

6.2.5 Statistical analysis

The effect of treatment and time on cloacal body core temperature and IRT body surface temperature measurements was investigated using general linear mixed models (package *lme4* in R version 3.4.1). We analysed the cloacal body core temperature response and the IRT body surface temperature response to treatment (LPS challenge or saline control), with cloacal or IRT body surface temperature in each ROI (eye, comb, head, wing, leg, whole-max) as the response variable, and time and treatment (LPS challenge or saline control) as explanatory factors. To test whether the cloacal and body surface temperature responses to the treatment were non-linear over the time course of the study, a quadratic term for time was also applied. As there were multiple measures from the same bird, and multiple birds were housed in the same pen, pen and bird identity were used as random factors in the model. Starting from the full model a backward stepwise model simplification approach was used by dropping the least significant term one by one, starting with interactions; likelihood ratio tests were used to compare between the models. A general linear mixed model (package *lme4* in R version 3.4.1) was also used to test whether the IRT body surface temperature in each ROI was correlated with the cloacal body core temperature. As there were multiple measurements from the same bird over time, pen and bird identity were entered as random factors, and as the baseline temperature may have changed over time, it was also necessary to take the time when the temperature was measured into account. The interactions of each IRT surface temperature response with time and treatment were also included to test whether the relationship between cloacal and each IRT surface temperature measurement differed with time and between treatment groups. Two-tailed P-values <0.05 were considered statistically significant.

6.3 Results

6.3.1 Cloacal and surface temperature measurements

Cloacal temperature in the control and LPS challenged birds at the start of the experiment reflected normal body temperature, ranging between 40.2 and 41.5°C. Over the time course of the study the mean cloacal temperature of the saline control group fluctuated but did not exceed a maximum of 41.2°C. As expected, cloacal temperature in the LPS challenged group became elevated at 6h and 12 h post treatment (Figure 6.2) reaching a peak mean value of 41.8°C at 12 h post-treatment. A recovery phase then followed during which the temperature decreased over the next 12 h to return to baseline levels. This was consistent with ad hoc observations of bird health status that indicated that the demeanour of the LPS challenged birds had returned to normal within 24h.

The increase in cloacal temperatures in the LPS group was mirrored by body surface temperature measures captured by IRT. Mean and SEM body surface temperature measurements for both treatment groups (LPS challenged or saline control) are presented in Figures 6.2 and 6.3 (A-F). Note that at 3 h and 9 h post treatment data sets are available for the body surface temperature measurements only.

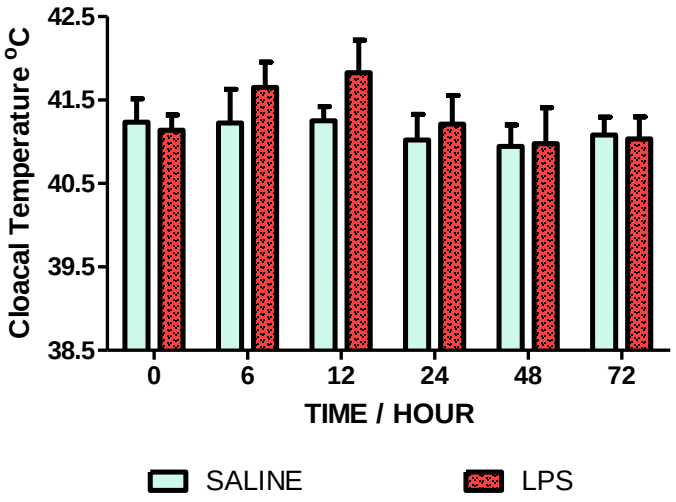

Figure 6.2 Cloacal temperature variation over 72 h following LPS treatment.
Mean and SEM surface temperature measurements in LPS and saline control bird pre (0h) and post treatment (3, 6, 9, 12, 24, 48 and 72h). The red bars represent the LPS treated group and the green bars the saline control group.

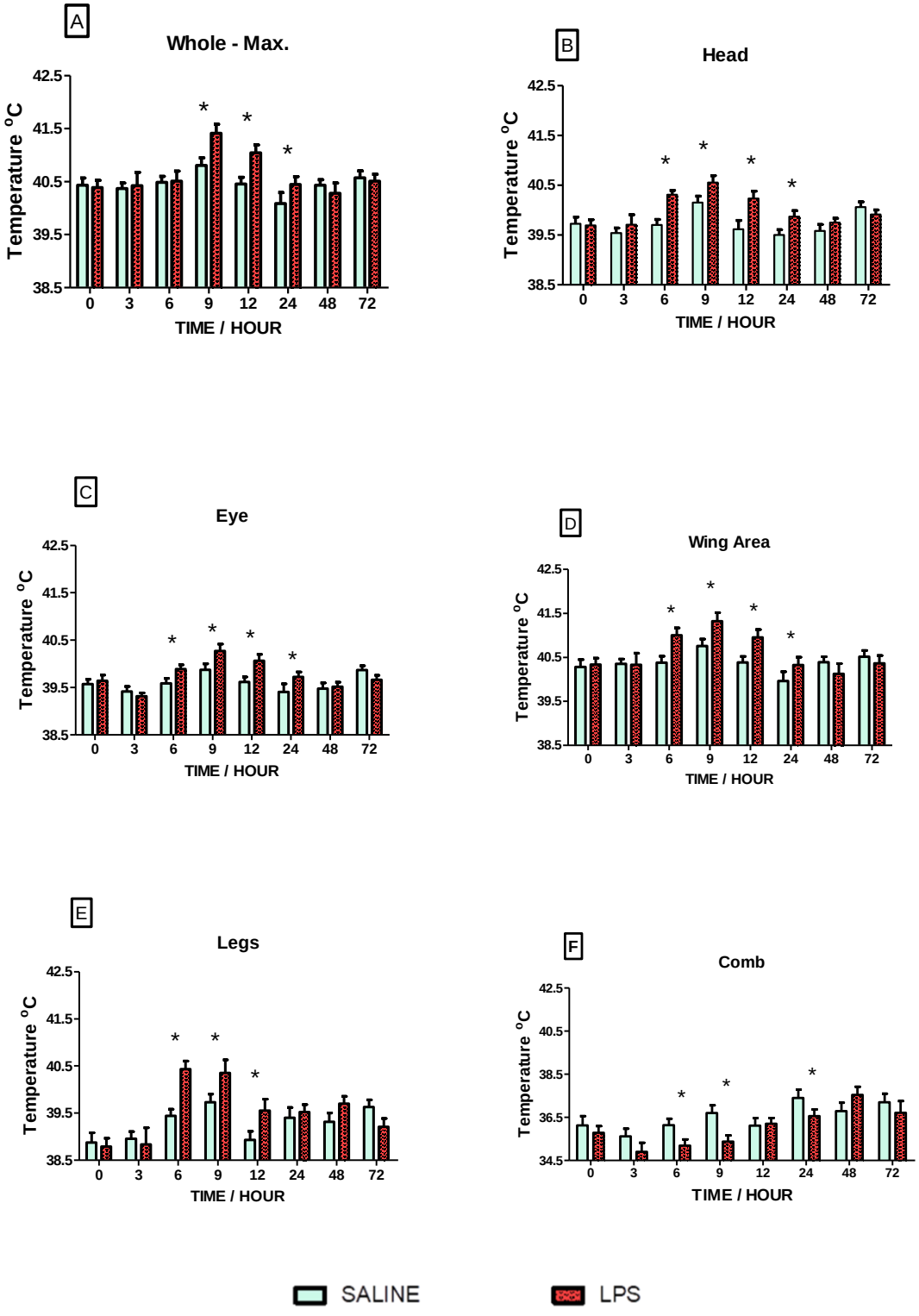

Figure 6.3 Surface body temperature measured by IRT.
Mean and SEM for surface temperature measurements in LPS and Saline Control bird pre (0h) and post treatment (3,6,9,12,24,48 and 72h). The red bars represent the LPS treated group, the green bars the saline control group. Significance of deference is indicated * P<0.05.

At the start of the experiment, body surface temperature measurements were at least 1°C lower than the cloacal temperature measurements (Fig 6.3). Surface

temperatures in the saline control group fluctuated over the time course of the study irrespective of the ROI (Fig 6.3). In the LPS challenged group (with the exception of the comb region), an increase in temperature was observed at 6h (head, eye, wing area and leg) and 9 h (whole-max) post challenge which was consistent with the onset of pyrexia as indicated by cloacal measurements.

The results of the general linear mixed models for the effect of treatment (LPS or control) and time (linear and non-linear) on cloacal temperature and surface temperature measurements are presented in Table 6.1. A significant linear interaction (P=0.011) between treatment and time was observed for cloacal temperature. According to the model the cloacal temperature declined linearly throughout the 72-h measurement period, but as anticipated the decline was steeper in the LPS challenged group than in saline control group. The nonlinear model did not reveal any significant effects of treatment or time or interactions on cloacal temperature. The general linear mixed models also revealed significant interactions between treatment and time for comb, wing and image–max body surface temperature measurements, again with a steeper linear decrease over time in LPS challenged birds compared to control birds (Table 6.1 (e-g)). An additional quadratic effect of time was revealed for the comb surface temperature measurement, but this did not differ between treatment groups (Table 6.1e). Significant nonlinear interactions between treatment and time were found for the leg, head and eye surface body temperature measurements (Table 6.1b-d) with the LPS challenged group displaying a steeper increase in body temperature up to a maximum temperature reached after 6-9 h post challenge which then declined more slowly than in saline control birds.

Table 6.1 Treatment and time effect on cloacal and surface temperature.
Results of General linear mixed models considering the effect of treatment (LPS challenged or control) and time (also nonlinear) on cloacal temperature (a), and body surface temperature measurements captured by IRT: (b) leg, (c) head, (d) eye, (e) comb, (f) wing area and (g) whole-max . Pen and bird identity were random factors. Statistically significant effects are in indicated by bold text

Temperature measurement	Treatment	Time	Treatment*Time	Time^2	Treatment*Time^2
(a) cloacal	χ_1^2=4.01	χ_1^2=0.50	**χ_1^2=6.53**	χ_1^2=1.37	χ_1^2=2.20
	P=0.045	*P*=0.044	***P*=0.011**	*P*=0.242	*P*=0.138
(b) leg	χ_1^2=3.66	χ_1^2=13.07	**χ_1^2=3.98**	χ_1^2=11.26	**χ_1^2=6.83**
	P=0.055	*P*<0.001	***P*=0.046**	*P*<0.001	***P*=0.009**
(c) head	χ_1^2=2.71	χ_1^2=0.02	χ_1^2=3.26	χ_1^2=1.53	**χ_1^2=6.15**
	P=0.100	*P*=0.891	*P*=0.071	*P*=0.217	***P*=0.013**
(d) eye	χ_1^2=1.53	χ_1^2=0.09	χ_1^2=3.45	χ_1^2=0.40	**χ_1^2=6.31**
	P=0.216	*P*=0.769	*P*=0.063	*P*=0.529	***P*=0.012**
(e) comb	χ_1^2=2.92	χ_1^2=41.50	**χ_1^2=4.68**	**χ_1^2=21.66**	χ_1^2=2.53
	P=0.087	*P*<0.001	***P*=0.030**	***P*<0.001**	*P*=0.112
(f) wing area	χ_1^2=1.32	χ_1^2=5.43	**χ_1^2=9.12**	χ_1^2=1.28	χ_1^2=1.05
	P=0.250	*P*=0.020	***P*=0.003**	*P*=0.257	*P*=0.307
(g) whole-max	χ_1^2=2.88	χ_1^2=5.50	**χ_1^2=7.19**	χ_1^2=1.18	χ_1^2=1.88
	P=0.090	*P*=0.019	***P*=0.007**	*P*=0.278	*P*=0.171

6.3.2 The relationship between the surface temperature of different body regions and cloacal temperature

The results of the general linear mixed models relating cloacal temperature to each body surface region in LPS challenged birds and saline control birds is provided in Table 6.2. Differences in the relationship between treatment groups (LPS and saline control) are also indicated. Scatter plots demonstrating and comparing the relationship between the cloacal temperature with body surface temperature measurement for each treatment group (LPS challenged and Saline control) are also presented in Figure 6.4 (A-F).

Table 6.2 Correlation body surface and cloacal temperature.
Results from general linear mixed models relating body surface temperature measurements captured by IRT with cloacal temperature in LPS challenged and control birds. Differences in the relationship between cloacal and IRT surface temperature measurements between treatment groups (LPS and saline control) were tested with the surface temperature-by-treatment interaction; all surface temperature-by-time interactions were non-significant (all P > 0.336); all surface temperatures varied between time points (all P < 0.001). (* P < 0.05, ** P < 0.01, *** P < 0.001).

IRT body surface temperature measurements (ROIs)	Correlation between cloacal and IRT body surface temperature measurements (r^2)		Pattern in LPS and saline control groups (IRT body surface temperature-by-treatment interaction)
	Saline control group	LPS challenged group	
Leg	0.17*	0.46***	χ_1^2 = 2.94, P = 0.086
Head	0.16*	0.65***	**χ_1^2 = 17.79, P < 0.001**
Eye	0.26**	0.67***	**χ_1^2 = 13.67, P < 0.001**
Comb	0.02	0.04	χ_1^2 = 0.92, P = 0.336
Wing area	0.13	0.61***	**χ_1^2 = 8.71, P = 0.003**
Whole-Max	0.16*	0.70***	**χ_1^2 = 14.89, P < 0.001**

The image-max (r^2=0. 70), eye (r^2=0.67) and head (r^2=0.65) body surface temperature measurements were all strongly correlated with cloacal temperature (P<0.001) in the LPS challenge group. A significant correlation was also observed between the wing area and the cloacal temperature (r^2=0.61; P<0.001) in the LPS challenge group. The relationship between all of these body surface temperature measurements and cloacal temperature and were significantly weaker in the control group. The leg surface measurement was correlated to a greater or lesser degree with cloacal temperature in both treatment groups whilst the comb surface measurement showed no correlation with cloacal temperature in either the LPS challenge or saline control groups.

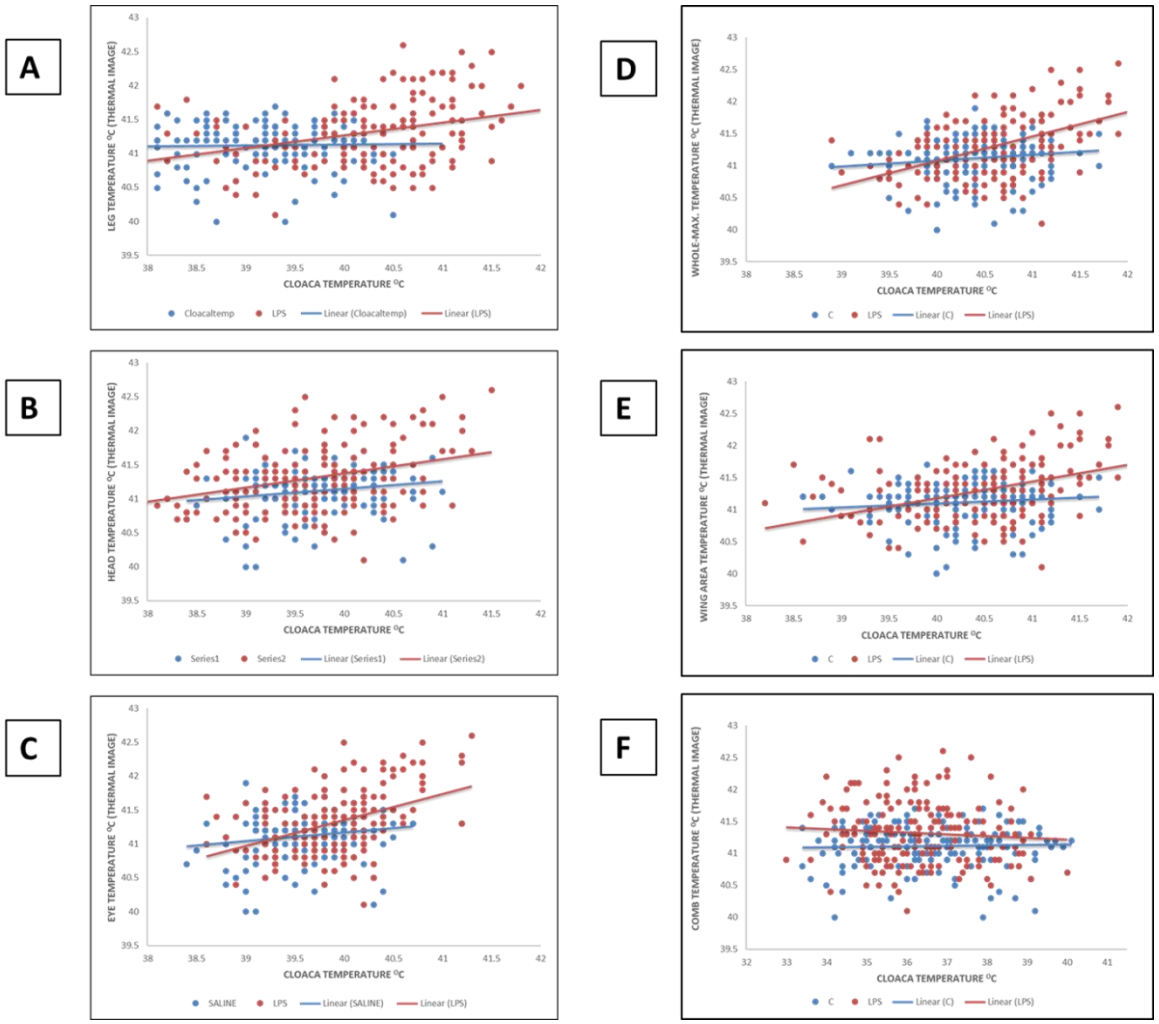

Figure 6.4 Relation between cloacal and surface temperature.
Scatter plots showing all measurements made on LPS challenged (red) and control birds (blue) to demonstrate the relationship between different body surface temperature measurements and cloacal temperature. A) leg , B) head, C) eye region, D) image-max E) wing area and F) comb.

6.4 Discussion

The aim of this study was to establish if IRT has potential as a non-invasive method to detect the early onset of pyrexia in broiler chickens, using the model of an LPS challenge. The objectives were to a) identify, b) establish the relationship between body surface temperature changes in different regions and cloacal temperature in both normal and pyretic individuals. These findings show that body surface temperature measurements made using IRT can indeed be used as a non-invasive method to detect the early onset of pyrexia in broiler chickens following an LPS challenge. At 6 h post treatment the cloacal temperature increased in the LPS challenged birds, which is consistent with the onset of pyrexia as described in other LPS challenge experiments (e.g. Cheng et al., 2004). Corresponding increases in body surface temperature were detected using IRT in five out of the six ROIs analysed in each image (head, eye, leg, wing area and image-max). Between 6 and 9 h post treatment the temperature increased in each of these ROIs, before decreasing as the birds recovered.

The linear GLM models used to analyse the data revealed a significant interaction between treatment and time for cloacal temperature and for four of the body surface regions (leg, comb, wing and image-max). This model predicted that for each of these measurements the temperature declined more rapidly in the LPS challenge group than in the saline controls. For the leg, eye and the head a significant non-linear interaction was observed between treatment and time. In this case, the initial body surface temperature increased reaching a maximum at 9 h post treatment. Again, this pattern of change was more pronounced in LPS challenged birds. These results suggest that the surface temperature of the leg, eye region and head are most sensitive to the changes associated with pyrexia. The lack of available data for cloacal temperature at 3 h and 9 h could explain the lack of significance when the non-linear model was applied to this measurement.

The cloacal temperature was used in this study as a proxy for body core or deep body temperature, as in previous studies (Huggins et al.,2012; Zehner et al., 1991). Whilst it is recognised that internal temperature is most accurately determined using surgically implanted loggers or transmitters and gastrointestinal or non-surgically placed devices, cloacal temperature is a less invasive alternative

which is convenient when the subject can be easily captured and restrained (McCafferty et al., 2013). The stress of capture and indeed the temperature measurement itself can bring about stress-induced hyperthermia (SIH) mediated by the sympathetic adrenal and the hypothalamic–pituitary–adrenal (HPA) axes, as well as through increased metabolic heat production associated with the escape response (Bouwknecht et al., 2007; Busnardo et al., 2010). In the current study the mean cloacal temperature of the saline control group fluctuated by approx. 0.5 ^{o}C over the time course of the study. The higher temperatures observed in the first 12 h are most probably attributable to the stress of being repeatedly handled and disturbed, but were small compared to the magnitude of change seen in the LPS challenge group. Compared to cloacal temperature, body surface temperature (image-max.) was generally lower by 1.1 and 0.8^{o}C at 6 and 12 h respectively in the LPS chickens. As anticipated the temperature of the eye region which is closest to the brain didn't fluctuate as much as some of the other body surface regions in the saline controls. The inner canthus of the eye is hottest part of eye region (Renshaw et al., 2011) and is the surface region which is often closest to core temperature compared to other peripheral regions. The eye and the head ROIs were useful for detecting temperature changes associated with the onset of pyrexia in the LPS challenged group. Both regions were also strongly correlated with the cloacal temperature (Table 6.2). The image-max measurement which corresponds to the maximum temperature of the whole IRT image was also able to detect the onset of pyrexia and was highly correlated with the cloacal temperature. Given the closeness of the R2 values for the head, whole max area and eye, it can be concluded that the head region in general is good for detecting the onset of pyrexia using IRT. This concurs with Giloh et al., (2012), who previously reported a strong correlation between IRT measurements of the facial area and cloacal temperature in broiler chickens exposed to different environmental temperatures. Under both acute and persistent heat exposure these authors found that both peripheral and facial surface temperature increased until a new thermodynamic balance was achieved, with heat being carried from the core to the surface by the circulation and then dissipated into the environment as a result of the increased temperature gradient. Giloh et al., (2012) concluded that facial surface temperature was a sensitive indicator for heat stress intensity and metabolic status in broiler chickens. The current study suggests that the surface temperature of the head region is also a good candidate for monitoring the febrile response of chickens under challenge conditions.

The leg region was also captured the onset of pyrexia but in this case the IRT measurement did not correlate well with the cloacal temperature in real time (P value). This was not unexpected since the legs are peripherally located in comparison to the other ROIs. Regarding the wing area as a potential site to measure body surface temperature changes associated with pyrexia, in this case the amount of feathering will determine if this area can be used effectively. In the context of the current study the birds were only 3weeks of age and patches of bare skin were still present in and around the ROI designated as the wing area in older birds, insulation from feathers is likely to limit the usefulness of the wing region as a thermal window.

The chicken comb is richly vascularized in its superficial dermis and is involved in temperature regulation (van Kampen, 1971; Stettenheim et al., 2000). Changes in comb temperature due to vasomotor responses have been recorded with infrared thermography (Edgar et al., 2011; Herborn et al., 2015). Furthermore, comb temperature has been applied as a welfare indicator associated with environmental choice in laying hens (Nicol et al., 2009). In the current study, the comb, which is still relatively small in 3 weeks old broiler chickens, did not dissipate heat well and was the least predictive region for detecting pyrexia in this study. In adult chickens both the comb and the wattles are known to play an important thermoregulatory role during exposure to high environmental temperatures (Moe et al., 2017) and so this finding varies with age. The comb was implicated in heat dissipation following stress induced hyperthermia in 23 weeks old laying hens with large combs (Herborn et al., 2015). Further work is therefore necessary to determine if the comb for example is an important site for detecting the early onset of pyrexia in birds of different ages.

In order to get accurate measurement with the IRT, it was crutially important to keep same distance of the camer from each chaptured chicken and at specific orientation.. This was to reduce variability in this proof of principle study. Such controlled conditions may be difficult to replicate in freely moving birds under commercial conditions, so we are still some way away from being able to apply these results on a larger scale. Nevertheless, using thermography to detect the early onset of pyrexia is potentially still considerably easier and potentially less costly than other serological tests. As thermographic equipment becomes increasingly affordable, then it may not be too long before automated cameras

systems become available. The challenge then will be how to tease apart body surface temperature changes resulting from exposure to disease from those associated with chronic and acute stress. These results show that IRT can be used to detect surface temperature changes associated with pyrexia following an LPS challenge in broiler chickens and provide crucial information on optimal areas for candidate thermal windows. Further work must determine if body surface changes associated with pyrexia are dependent on the type of febrile illness, and the extend to which methodology must be adapted to accommodate different species, genetic strains and ages poultry.

Chapter 7 General Discussion

7.1 Responses of APP

Acute phase proteins are quantifiable mediators of the APR and have been used as an indicator of human (Blackburn, 1994) and veterinary (Gruys et al., 2005) health. However, the limited availability of ELISA kits to detect these APP has restricted the information to just a few APP. This thesis has mainly focused on four APP: SAA, AGP, CP, and OVT. These APP were chosen because of their significant anticipated role as well as the recent availability of commercial ELISA kits to detect three of them in blood samples. The performance of these chicken ELISA kits for measuring serum concentration of AGP, SAA and CP were acceptable for use and can give valid results in the chickens' samples as the results have shown in Chapter 2. For the future work it would be of interest to study the effect of heating on the performance of the SAA kit. As this APP is associated with the lipid, the influence of heating on the dissociation of SAA could potentially have a significant impact on its antigenicity and therefore is reported concentration.

To study the APP response, the chicken's immune system was independently challenged by a Newcastle disease and Infection bronchitis vaccine (N/B) (chapter 3), in this chapter AGP, SAA, CP and OVT in addition to H/L ratio where investigated before and after vaccination. On the other experimental work wherepoultry Red Mite (PRM) influence and investigation AGP, SAA and CP where measured before and after challenge (chapter 4). In the last experiment challenge with LPS *E. coli* to investigate its effect on the AGP, SAA, CP and OVT also H/L have monitored and the IRT to link the surface temperature variation with cloacal temperater changes as a result to the inflammtore response to LPS (Chapter 5).

Among the four types of APP used, there were clear difference in the level of responses and the timing. SAA changed by the highest magnitude and gave earlier responses to stimuli comparison to another APP. This confirms the opinion of others, that SAA is a major APP in chickens (Ceron et al., 2005; Ceciliani et al., 2012). SAA increased by 2, 5, and 30-fold following the administration of N/B vaccine (Figure 3.3), PRM (Figure 4.4), and LPS E. coli (Figure 5.8), respectively. These differences could be attributed to the fact that different levels of cytokines were released according to the pathogenicity of the challenge whether disease or

other insult (such as vaccination) and of course the host's response to these (Jergens et al., 2003). This is in turn would consequently affect the level of APP response (Eckersall, 1995; Ceron et al., 2005). These differential alterations in responses of APP could occur for various reasons. First, different co-stimulatory cytokines could be secreted depending on the underlying stimuli (Gabay and Kushner, 1999; Bode et al., 2012). This may cause varying hepatic APP expression rates, and production of APP. Differences in response of haptoglobin (Hp) and SAA to primary and secondary vaccinations indicate that the cytokine driven APP response mechanisms vary, not only with the individual but also with immune status (Eckersall et al., 2008). Among species there are differences in homology of APP at the level of the gene and amino acides sequences, for instance SAA in hourses showed similarty in homology 80.6% with dog, 76.9% with human and 71.9% with duck (Ma et al., 2000). This homology variety could explain alteration in level responses of APP. Secondly, not only gene expression, but also all subsequent processes, such as post-transcriptional regulation plays a substantial role in induction of some, but not all APP such as SAA (Jiang et al.,1995). Secretion efficiency may also be influenced by other stimuli (Gabay and Kushner, 1999). A third mechanism could be attributed to the differential metabolism and clearance of APP (Schrodl et al., 2016). In such cases, the clearance mechanism is differentially regulated by how long the APP remain in circulation which depends on their half-life (Uhlar and Whitehead, 1999; Gollaher and Bausserman, 1990). Whereas a fourth aspect leads to the question whether different extrahepatic tissue origins should be taken into account as supplementary sources of circulating APP.

It is thought there is a relationship between APP in liver synthesis and the extra hepatic expression (Schrodl et al., 2016), which could have both a functional and a diagnostic significance. The diagnostic importance would be the ability to quantify and attribute APP to specific sources (Schroedl et al., 2016). This assumes that firstly, extrahepatic APP have different moieties and secondly, that they contribute to plasma APP alterations as a result to stimuli.(Schroedl et al., 2016). It is probable that the widely observed heterogeneity of circulating APP (glycosylation, isoelectric points, and chromatographic distribution), and their correlation to various sources, could provide valuable contribution as biomarkers of inflammation (Schrodl et al., 2016). The functional importance would be that the dual secretion of APP could interlink local and systemic reactions.

The available evidence suggests that the focal and extra-hepatic expression of several APP can be detected in healthy and pathological cases (Kalmovarin et al.,1991; Berg et al., 2011, Marques et al. 2017). Generally, extra-hepatic expression rates are low in comparison to that of the liver in healthy individuals, but this rate increases dramatically under focal injury (Reinhardt et al., 2013). The current investigation showed that mRNA expression of APP can be measured in a range of extra hepatic tissue including the lung, gizzard and adipose tissue in both saline control and LPS treated chickens (Figures 5.14-5.17). In general, the level of expression of APP was low, even in the LPS *E. coli* treated chickens. This is most likely because of the late collection time of tissue compared to when the stimulus was administered (72 h post treatment). By this time the chickens were in recovery stage, and synthesis of APP had probably returned to the healthy level. In the current study because of lack of the resources, it was only possible to harvest tissues at the end of the experiment (72 h). Ideally a subset of birds from both the LPS and control groups would have been euthanized at 0, 6, 12, 24, 48h.

On the other hand, it would be of interest to measure APP expression in chicken's samples obtained from farms or abattoirs for detection extrahepatic expression of APP at different pathological cases.

This finding suggests that extrahepatic tissues of SAA could be contributing to the circulating plasma levels of SAA during the recovery period, also SAA had higher rate of plasma level and higher extrahepatic expression which indicate it is indeed a major APP in chickens.

7.2 Other parameters of APR

Several procedures have been utilised in the current investigations of APR. These main procedure, following validation, was ELISAs assays to estimate APP (SAA, AGP and OVT) (Chapters 3,4,and 5) as well as immunodiffusion assay (OVT) Chapter 3 and 5. Where appropriate, additional techniques were employed such as electrophoresis of proteins followed by densiometric analysis (chapter 4), protein identification by mas spec proteomics (Chapter4) and Western blotting to assess APP 3 and 4. Whilst, in other experimental study real time PCR (what gene mRNA?) have been used (Chapter 5). Besides investigation APP, and in order to broaden innate immune responses investigation, other blood components have

been assessed such as, measuring corticosterone concentration in blood by ELISA, H/L ratio by use blood smears to assess differential white blood cells count, and monitoring fever development by use thermal imaging camera (IRT).

The H/L ratio also is commonly used to investigate stress in birds, particularly chickens (McFarlane and Curtis, 1989; Maxwell et al., 1992; Puvadolpirod and Thaxton, 2000; Post et al., 2003). In the N/B vaccinated or LPS challenged birds, a change in H/L ratio was observed from 0.11 to 0.54 (chapter 3, Figure 3.1), and 0.03-0.27 (chapter 5, Figure 5.6), respectively. The change of the H/L ratio in other studies ranges from 0.19 to 64.67 in response to different stimuli (Clark, 2015).

Corticosterone blood concentration was selected as an additional parameter to measure in the LPS challenge experiment. In addition to its role as a metabolic hormone, corticosterone is one of the major mediators of the stress response in vertebrates (Siegel, 1985, 1995; Khansari et al., 1990; Husband,1993; Curtis, et al., 1980). Within minutes of the perception of a stressor, circulating concentrations rise dramatically (Jones et al., 2016). In the current investigation, corticosterone was elevated by 3.5-fold in LPS *E. coli* chick group, and was also significantly higher than the control group, at 6 h. It then decreased to baseline levels (Figure 5.5). The level of corticosterone in previous studies is reported to have increased by 2.5-fold in responses to PRM challenge (Kowalski and Sokot, 2009) and in another study by 5-fold (Nakamura et al., 1998). Corticosterone plays a multifunctional role through alteration of endocrine and metabolic factors, including leukocytes and immune mediators (Shini et al., 2008). Thus, corticosterone is stimulated by anti-inflammatory cytokines and downregulated by pro-inflammatory cytokines to establish a balance after onset of the stimuli (Elenkov and Chrousos, 2002). However, in the current work corticosterone was shown not to be a reliable parameter to monitor the APR because it does not last for any length of time compared to APP or H/L ratio (see below), furthermore, it is expensive to measure by ELISA kit.

The thermal imaging a non-invasive method has been used to detect body surface temperature changes in birds for many years (McCafferty, 2013). For example, a

rapid drop in skin temperature in chickens has been shown to be a good indicator of stress (Herborn et al., 2015). In this thesis this technique was used to investigate the onset and development of pyrexia after LPS challenge (Chapter 5). This technique has been applied in other animals, e.g in cows (Paulrud et al., 2005), in horses (Fonseca et al., 2006), as well as chickens (Wilcox et al., 2009). The application of this technique to detect the onset of pyrexia in chickens in response to LPS challenge is novel and was shown to be sensitive enough to detect a temperature change of less than 1^{o}C in body surface temperature which was sufficient to discriminate between pyretic and healthy birds. The results have additionally indicated a robust correlation between thermal imaging and the cloacal temperature (Table 6.2). Consequently, it is suggested that thermal imaging can be adopted as a useful and non-invasive tool to detect pyrexia in chickens. It represents a technology that could adapted as a non-invasive monitor of health in farms.

7.3 Conclusion and Future work

This project has been based almost exclusively on in vivo studies using chickens. Based on ethical and the three R principles, there was great concideration given to the number of birds that could be handled, blead and ethanased in a human and efficient manner. As a consequence one of the main limitation of this study was the number of chickens have been used at each collecting time points although at the study desing stages it was anticipated that the number in each group was statistically valid for these type of studies. The consumable budget was restricted and given the cost of the invivo studies, there was limitations on the extent of the proteomic study which has great potential for identifying candidate bionmarkers, especially in the LPS experiment. For future work it would be of interest to investigate if there is a link between the APP response and vaccine efficacy. For example, by administering different vaccines for a particular pathogen to different groups of chickens, and measuring the APP at 0, 10, 15 and 20 d post vaccination the relationship between the APP and final Antibody titre for each vaccine could be determined (Figure 7.1). Such a study could supply data to link the early responses of (SAA, H/L ratio and IRT) and late Ab titre, and characterised how for example SAA (the most sensitive APP) could be used as a tool to help define the efficacy of each vaccine.

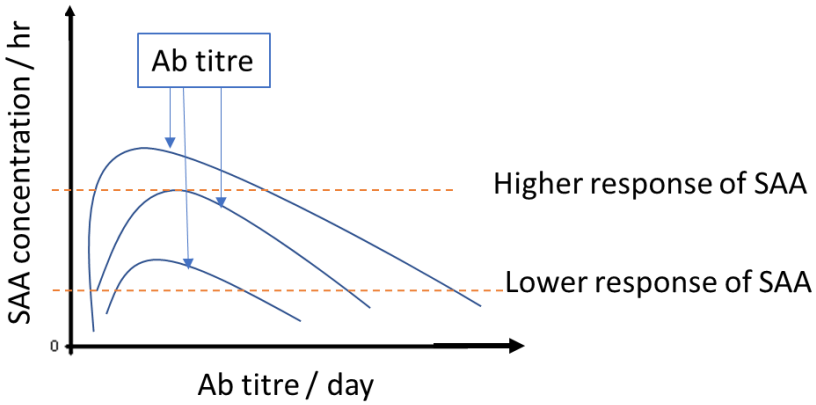

Figure 7.1 A diagram illustrates a hypothesis of relationship between response of SAA and Ab titre, to utilise it as a tool to estimate efficacy of vaccine by link the early SAA response to the Ab titre level.

The relationship between the systemic and the local expression of APP may be of valuable diagnostic indicator. It is highly probable that the widely observed heterogeneity of circulating APP is related to a variety of sources and if they can be identified specifically and quantified they could in themselves become valuable biomarkers (Ray et al., 2014). Possibly, extra-hepatic APP are structurally adapted to local activities (Inforzato et al., 2006). To broaden the scope, it is necessary to examine the extra hepatic expression of APP of tissue and look for the presence of APP in different body fluids. e.g. bronchial fluids or saliva. Thus, could help to support both diagnosis and prognosis of diseases in poultry.

For the future work, thermal imaging for detection of further subclinical cases and its use alongside APP especially SAA is worth investigation as a way of defining subclinical diseases in chickens more speedily and accurately.

One would hope that this study will help to stimulate a dialog between avian immunologists and pathologists that results in coordinated and integrated approach to produce more practical diagnostic, prognostic and prevention solutions that will benefit the industry and improve the well-being of commercial poultry.

Proteomic is advancing knowledge at a remarkable rate (Eckersall and McLaughlin, 2011). It could be that there are as yet many undiscovered APP in the blood sera those we are currently measured of the field of APP is set to expand the potential use of some of the these as biomarker for early disease detection as its infancy.

Appendixes

Appendix A: - Publications

Acute phase proteins and stress markers in the immediate response to a combined vaccination against Newcastle disease and infectious bronchitis viruses in specific pathogen free (SPF) layer chicks

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ABSTRACT Vaccination is an important tool in poultry health, but is itself a stressor often resulting in a reduction in feed intake, body weight gain, and nutrient digestibility. In other species, vaccination is associated with an immediate acute-phase response. As an important immune parameter, the circulating heterophil/lymphocyte (H/L) ratio is a well-recognized parameter of stress in poultry. In this study, the effects of a routinely used commercial poultry vaccine on the acute phase response (APR) and H/L ratios in specific pathogen-free (SPF) layer chicks was examined to determine if post vaccination (PV) stress and an APR occur. A combined Newcastle disease and infectious bronchitis vaccine (Nobalis Ma5+Clone 30) was administered to SPF chicks by the intraocular route at age 7 d. Acute phase proteins (APP), alpha-1 acid glycoprotein (AGP) and serum amyloid A (SAA) were measured by enzyme-linked immunosorbent assays at d 0 (pre-vaccination) and d 0.5, 1, 2, 3, 4, 5, 6, and 21 PV. Stress was determined in the chicks by measurement of the H/L ratio. The immune response to the vaccine was estimated by measurement of the antibody (IgY) response to the vaccine at d 21.

The antibody titer was significantly ($P < 0.05$) higher in the vaccinated group at 21 d PV, confirming stimulation of the immune system. The H/L ratio was also significantly higher in the vaccinated group at 1 to 2 d ($P < 0.01$) and at 3 d ($P < 0.05$) PV. The concentration of SAA increased by 2.8-fold, from 63.7 μg/mL in controls to 181 μg/mL in the vaccinated group, ($P < 0.05$) at 1 d PV. AGP increased 1.6-fold at 2 d PV, (from 0.75 g/mL in the control group to 1.24 g/mL in the vaccinated group, $P < 0.05$).

In conclusion an immediate but mild APR occurred in the chicks following intraocular vaccination, whereas the stress response as measured by H/L ratio seemed to be more specific and sensitive. Measurement of these biomarkers of the host response could be a tool in vaccine development.

Key words: chicken, acute phase, vaccination, stress

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INTRODUCTION

Acute phase proteins (**APP**) are a group of blood proteins involved in restoring homeostatic balance by restricting growth of microorganisms, mediating the inflammatory response and the effects of stress through an antibody-independent response. The APP are also considered to have diagnostic and prognostic potential because of the correlation between their concentration in blood and the response of the host to infection or inflammation (Cray et al., 2009). There is increasing interest in APPs in chickens as a physiological marker for health and welfare, including infection and intriguing vaccine response to both bacterial and viral pathogens (O'Reilly and Eckersall, 2014). Responses of APP in terms of vaccination stimuli have been investigated in other species; for instance, in horses (Andersen et al., 2012), sheep (Eckersall et al., 2008) and calves (Arthington et al., 2013). These previous studies reported an acute phase response to a variety of vaccines and therefore suggest the possibility that monitoring the APP may be a means to determine the efficacy of a vaccine in stimulating the innate immune system and as such could be a tool of value in vaccine development.

In chickens, alpha-1 acid glycoprotein (**AGP**) responds as a moderate positive APP (Chamanza et al., 1999; O'Reilly and Eckersall, 2014) following experimental infection. Investigation of the plasma AGP response, in White Leghorn SPF chickens at age 3 wk old, inoculated by an intraocular route with a highly virulent strain of Gumboro disease virus, showed a 4.6-fold increase in serum AGP concentration at 2 d post immunization, which peaked at 6 d at 6.2-fold the pre-treatment level. In contrast, inoculation with an

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attenuated strain of Gumboro disease virus lead to a peak in AGP of 2.4-fold at d 2 post inoculation (Inoue et al., 1997). AGP levels have also been shown to increase significantly ($P < 0.05$), at 12 to 48 h post IV injection with lipopolysaccharide (**LPS**) from *Escherichia coli* in male broiler chickens at 3 wk old (Takahashi et al., 1998)

Inoculation of commercial layer chickens with *Mycoplasma gallisepticum* vaccine caused a significant increase in serum AGP concentration, which started at 1 d PV(Peebles et al., 2014). In this study, the vaccine was administered via the intraocular route in one group and subcutaneous injection in another group. The concentration of AGP remained significantly higher, in both administration routes, than in the control group for up to 21 d post inoculation.

Serum amyloid A (**SAA**) is another major positive APP in chickens (Alasonyalilar et al., 2006) and is immunomodulatory, inhibiting pyrexia and down regulating pro-inflammatory events during an APR (Shainkinkestenbaum et al., 1991; Uhlar and Whitehead, 1999). However, due to the lack of commercial assay systems, there have been few reports on this APP in chickens and none in relation to post vaccination (**PV**) stimulation. In this study we were able to monitor SAA in chicken serum using a newly available species-specific enzyme-linked immunosorbent assay (**ELISA**) system and therefore determine if this major APP is also stimulated by vaccination.

Changes in white blood cell count, especially in heterophils/lymphocyte (**H/L**) ratio, have been used as a measure of stress in chickens (Gross and Siegel, 1983; Shini et al., 2008) (Crowther, 2009; Ohara et al., 2015). The H/L ratio of birds can be affected by health disturbance or stress (Crowther, 2009), including transport stress (Huff et al., 2005; Matur et al., 2016), possibly due to the transition of leukocytes from the marginal pool to peripheral circulation (Duncan, 1987).

In this study, we measured the AGP, SAA, and the H/L ratio in specific pathogen-free (**SPF**) chickens following routine vaccination via the intra-ocular route to determine whether the immediate post-vaccination innate immune or stress response was most sensitive to the mild or severe vaccination stress. Our specific aim was to determine if an immediate APP response occurs following intra-ocular vaccination as well as via the intra muscular route (Peebles et al., 2014) which would allow subsequent investigations on the pathophysiology of avian APPs and their interaction with the protection of the host provided by such vaccination. The vaccine chosen for the study, a combined Newcastle disease and infectious bronchitis (**N/B**) live, freeze-dried virus vaccine, is routinely used in chicken production, and this dual vaccine was selected to stimulate an APP response under conditions similar to those on commercial farms. Vaccination routes recommended for this vaccine are by spray, in drinking water or by eye drop. Intraocular vaccination (eye drop) was selected to ensure every individual bird received the same dose, which could not be guaranteed with the other routes. To evaluate the success of the vaccine, the amount of specific antibody (IgY) raised against the immunogen proteins of the vaccine after 21 d, was also determined.

MATERIAL AND METHODS

SPF Chicks and Housing

One hundred and eighty 1-day-old SPF White leghorn layer chicks were hatched out at the experimental farm (Cochno Research Farm, University of Glasgow) and divided into 2 batches each containing 90 chicks. Each batch of chicks was then placed in a separate, controlled-environment room (R1 and R2) in one of 2 pens (n = 45 per pen). The 2 pens in each room had a litter of wood shavings and were fitted with a brooding ring. The stocking density was 12 chicks/m^2.

The chicks were fed ad libitum with a commercially available chick crumb formulated to meet or exceed National Research Council (NRC, 1994) guidelines, and the birds had access to fresh water throughout the study period. The light, temperature, and ventilation within each room were automatically controlled and adjusted according to management guide recommendations. The chicks were allowed to adjust to their environment for the first 7 d before the experiment commenced. Strict biosecurity was applied to prevent cross contamination between the 2 rooms and each pen, not least the controls (R2) were always visited first and then the vaccinated room (R1).

Experimental Design

The experiment commenced when the chicks were 7 d old. There were 9 sampling time points; pre (d 0) and PV at 12 h, 24 h, and then 2, 3, 4, 5, 6, and 21 d thereafter. At each time point, 12 chicks were weighed and culled and samples collected, 6 per treatment and 3 per replicate pen. Full ethics approval was granted in advance by the University of Glasgow MVLS College Ethics Committee.

Vaccine and Vaccination

A commercially available combined Newcastle disease and infectious bronchitis (N/B) live, freeze-dried virus vaccine (Nobalis Ma5+Clone 30, MSD Animal Health) was used in this experiment. After re-constituting the vaccine in sterile saline solution, each dose contained at least $10^{3.5}$ EID_{50} of the IB strain Ma5 and 10^{6} EID_{50} Newcastle disease virus strain Clone 30.

The chicks in pens 1 and 2 in R1 were weighed and the vaccine was administered by the intra-ocular route with one drop applied to one eye. A sterile saline solution was administered by the same route to control animals in pens 1 and 2, which were housed in R2.

Blood Sampling and Assessment

At each sampling time point, 3 chicks were chosen from each replicate pen, weighed, then humanely culled by dislocation of the neck followed by decapitation. Following decapitation, approximately 1.5 mL of blood was collected from the major vessels in the neck using heparinized tubes. Fresh blood was used to make blood smears to determine the H/L ratio; the remainder was centrifuged (3,000 × *g*) for 15 min at 4 °C and the plasma immediately frozen at −20 °C. SAA and AGP levels were measured in all samples collected using commercially available ELISA kits described below. Plasma from samples on d 0 and 21 d were used to estimate the antibody titers as detailed below.

Heterophil/Lymphocyte Ratios

Differential white blood cell (**WBC**) counts were carried out on the blood smears stained with the May-Grunwald-Giemsa stain. Two hundred leukocytes were counted and classified per slide. The H/L ratio was calculated by dividing the total number of heterophils by the total number of lymphocytes (Gross and Siegel, 1983; Ohara et al., 2015). Samples used for this technique were at 0, 1, 2, 3, 4, 5, 6 d PV, and we dropped 12 h PV for technical issues.

Antibody Titer Raised Against the Vaccine

A modified antibody (**Ab**) titration assay (Snyder et al., 1983; Crowther, 2009) was used to determine the amount of IgY Ab raised against the vaccine in this study. A 96-well plate (Costar Assay Plate, Corning) was coated with the Newcastle and infectious bronchitis vaccine (Nobalis Ma5+Clone 30) diluted to a protein concentration of 20 μg/mL in 0.2 M carbonate bicarbonate buffer at pH 9.5 and incubated at 4 °C overnight. The protein content of the vaccine had been determined by a Bradford protein assay (Sigma Chem Co. Poole, UK) with bovine serum albumin as the standard. Each well was then aspirated and washed 4 times using Tris-buffer saline (**TBS**) 50 mM Tris-Cl, pH 7.5 containing 0.05% tween-20. Wells were blocked with 200 μL of 5% (w/v) Marvel milk protein diluted in TBS-T (0.05%) over 1 h at room temperature (**RT**) on a rocking plate. The plate was then washed as above.

Antibody standards were made by serially diluting pooled samples collected from vaccinated birds sampled at 21 d PV and by diluting 1:20 in TBS-T with 0.5% Marvel milk. This was standard 1 (**S1**) and given a value of 100 arbitrary units (**AU**) of antibody. It was then diluted in a 6-fold serial dilution to S6 (3.13 AU) using TBS-T with 0.5% Marvel milk. The chicken serum samples also were diluted 1:80 in TBS-T 0.5% Marvel milk. To duplicate wells a 100 μL aliquot of diluted standard or sample was added. After 1 h, incubation with constant shaking at RT, the plate was washed as above. The second antibody, anti-Chicken IgY VHH Single Domain Antibody conjugated to horseradish peroxidase (**HRP**) (Abcam, Cambridge, UK), was diluted to a concentration of 1:5000 with TBS-T 0.5% Marvel milk and added before incubating for another 1 h at RT by constant shaking, then washed as above. 100 μL of tetra-methylbenzidine (**TMB**, KPL Laboratories, Inc., Gaithersburg, MD) was then added to each well for 20 min at RT while rocking until a blue color developed, then 100 μL of stop solution (2 M H_2SO_4) was added. This caused the color to change to yellow. The absorbance of the resulting solution was measured at 450 nm using an OPTIMA absorbance microplate reader (BMG Labtech Ltd, Bucks, UK). A standard curve using 4-parameter fit curve was used to determine the antibody response.

Enzyme-Linked Immunosorbent Assays (ELISA)

The ELISA assays for chicken APPs were obtained from Life Diagnostics Inc., (West Chester, PA). They were performed according to the manufacturer's instructions with a dilution factor for the serum samples of 1:10,000 for AGP and 1:20 for SAA. Each individual sample was run in duplicate.

ELISA Assay for AGP

Diluted samples and standards were mixed thoroughly and 100 μL of each sample or standard was dispensed into duplicate wells of a 96-well microtiter plate. This was then incubated on an orbital microplate shaker at 150 revolutions per min (rpm) at RT for 45 min. Contents of the wells were then discarded and the wells were washed 5 times each using 1× wash buffer. After ensuring all residual droplets in the wells were removed by striking plates onto absorbent paper, 100 μL of the secondary antibody-HRP conjugate was then dispensed into each well and incubated on the shaker at RT for 45 min. The wash step was repeated and 100 μL of TMB reagent (HRP substrate) was dispensed into wells and a blue color development was allowed to proceed for 20 min on the shaker at RT. The reaction was stopped by adding 100 μL of stop solution per well into the wells. Absorbance was read using a FLUOstar Optima plate reader at 450 nm within 15 min of stopping the reaction. A four-parameter logistic curve (**4PL**) was used as described above. Intra-assay coefficients of variance (**CVs**) were 4.7% at 76.2 g/L ± 3.6 (mean ± SD) and 3.7%, at 32.6 g/L ± 1.48 (mean ± SD) (n = 40). Inter-assay CVs were 2.7%, at 43.6 g/L ± 1.17 (mean ± SD) and 5.8%, at 70.8 g/L ± 6.4 (mean ± SD) (n = 5) and the limit of detection was 1.7 g/L (3 SD from zero sample).

Table 1. Shows median ± SD of body weight for control and vaccinated groups at each sampling time. There were no significant differences by Mann-Whitney test, at any sampling time between the groups, n = 6 chicks per sampling time.

Age/d	Sampling time	Control Median (range)	Vaccinated Median (range)	*P* value
7	0	69.85 (56.5–84.2)	76.4 (60.8–81.2)	0.5752
8	1	77.55 (68.2–87.6)	78.6 (66.6–99.1)	1.0000
9	2	85.1 (77.8–97.1)	85.2 (79.4–94.3)	0.7488
10	3	92.7 (60.5–102.5)	95.45 (80.7–100)	0.0927
11	4	105.1 (90.4–122.3)	96.3 (58.9–109.6)	0.0929
12	5	105.35 (93.2–124)	103.5 (90.8–132.1)	0.4712
13	6	105.05 (96.6–138.9)	122.7(91.1–142.7)	0.2980
28	21	332.95 (294–390.7)	297.1 (271.5–387.9)	0.0957

ELISA Assay for SAA

Serum samples (50 μL) were first incubated in a heat block at 60 °C for 1 h to dissociate SAA from lipoproteins. Following heat treatment, the diluted samples and standard (100 μL) were incubated in the antibody-coated microtiter wells in duplicate together with HRP conjugate (100 μL) for 1 h. As a result, SAA molecules become sandwiched between the immobilization and detection antibodies. Contents of the wells were then discarded and wells washed 5 times each using 1× wash buffer. After ensuring all residual droplets in the wells were removed by striking plates onto absorbent paper, 100 μL of TMB Reagent was added and incubated for 20 min. Color development was stopped by the addition of 100 μL Stop Solution, changing the color from blue to yellow and the optical density was measured at 450 nm. A 4PL was used as described above. Intra-assay CVs were 5.7% at 57.5 μg/L 4.8 (mean ± SD) and 3.07% at 25.9 μg/L ± 1.8 (mean ± SD) (n = 40), inter assay CVs were 7.6% at 10.5 μg/L ± 0.6 (mean ± SD) and 5.3% at 103.7 μg/L ± 6.2 (mean ± SD) (n = 5) and the limit of detection was 0.21 μg/L (3 SD from zero sample)

Data Handling and Statistical Analysis

The antibody response to the vaccine, H/L ratios, and acute phase proteins (SAA, AGP) of the vaccinated and control groups, were compared at each sampling time point using a Mann-Whitney Test for non-parametric distribution (Minitab 17.1.0). In all analyses, $P < 0.05$ was used to represent statistical significance.

RESULTS

Chick Weights

The chicks in each group were within the same range, 69.85 g (56.5 to 84.2) median (range) for the control group and for the vaccinated group 76.4 g (60.8 to 81.2) median (range) at 7 d old. There were no significant differences in body weight between the groups in response to the vaccination (Table 1).

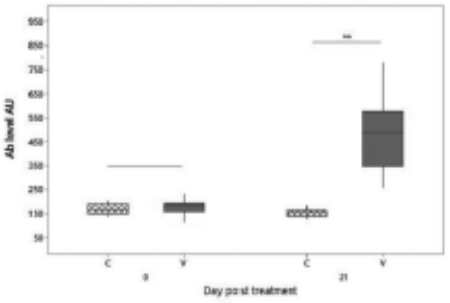

Figure 1. Antibodies levels pre-treatment 0 and 21 d post treatment in the control (C) (n = 6 per time point) and vaccinated (V) groups (n = 6 per time point). The difference was significant $P \leq 0.01$ at d 21 post treatment. (Horizontal bars with star indicate statistical differences between groups (** = $P \leq 0.01$). Data presented as box and whisker plots with median in the box, with 25 to 75 percentile range as the box and the whisker as 10 to 90 percentiles.

Antibody Titer Raised Against the Vaccine

The antibody titer was significantly elevated by 3.1-fold ($P < 0.01$) after 21 d to be 488AU (346 to 781) median (range) in the vaccinated group compared to the control group 154 AU (130 to 186) median (range) (Figure 1).

The Heterophil/Lymphocyte Ratio

The H/L ratio increased significantly ($P < 0.01$) to reach a peak of 0.58 (0.39 to 0.65) median (range) in the vaccinated group (**V**) compared to the control group (**C**) 0.20 (0.08 to 0.32) median (range) by on d-1 post treatment (Figure 2). This increase remained significant on d 2 ($P < 0.01$) and d 3 ($P < 0.05$) post treatment. From d-4 there was no significant difference in the H/L ratio between the control and vaccinated groups.

Acute Phase Proteins

AGP in the vaccinated group was statistically different from the AGP levels in the control group on d-2 post treatment. AGP increased 2-fold from 0.47 g/L (0.22 to 0.49) median (range) in the control group to1.007 g/L (0.45 to 1.39) median (range) in the vaccinated group,

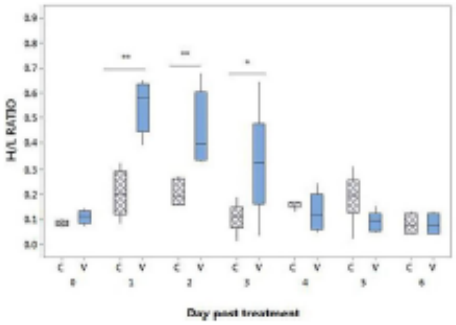

Figure 2. The heterophil/lymphocyte ratio was significantly higher in the vaccinated group (V) (n = 6 per time point) at 1, 2 and 3 d post treatment compared to the control group (C) (n = 6 per time point). Horizontal bars indicate statistical differences between groups (** = $P \leq 0.01$, * = $P \leq 0.05$). Data of 7 sampling time points are presented as box and whisker plots with median in the box, with 25 to 75 percentile range as the box and the whisker as 10 to 90 percentiles.

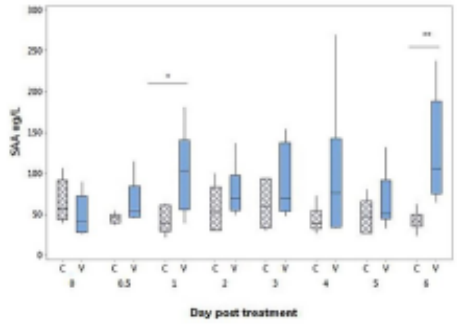

Figure 4. Comparison of SAA concentration in control (C) (n = 6 per time point) and vaccinated groups (V) (n = 6 per time point) over the time course of this study. Significant differences were detected (horizontal bars) at d 1 and d 6 post treatment, ** = $P \leq 0.01$, * = $P \leq 0.05$. Data of 8 sampling time points are presented as box and whisker plots with median in the box, with 25 to 75 percentile range as the box and the whisker as 10 to 90 percentiles.

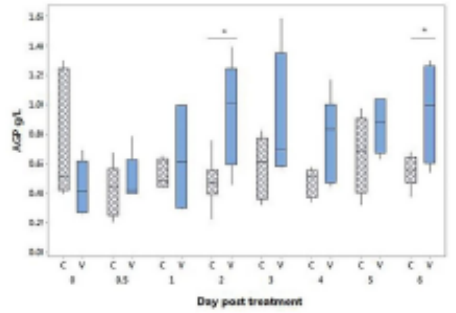

Figure 3. Comparison of AGP concentrations in vaccinated (V) (n = 6 per time point) and control groups (C) (n = 6 per time point) over the time course of this study. Significant differences (horizontal bars) were detected at d 2 and d 6 post treatment ($P < 0.05$). Data of 8 sampling time points are presented in median with 25 to 75 percentile range as the box and the whisker as 10 to 90 percentiles.

$P < 0.05$ (Figure 3). At d-3 post treatment the AGP levels were not statistically different from the control animals. At d -6 post treatment, another significant increase ($P < 0.05$) in the levels of AGP in the vaccinated group over controls was observed with a 1.9-fold increase from 0.54 g/L (0.37 to 0.68) median (range) in the control group to 0.99 g/L (0.53 to 1.29) median (range) in the vaccinated group.

SAA levels were significantly higher in the vaccinated group on d 1 post treatment ($P < 0.05$) with a 2.5-fold increase from 39.5 μg/L (20.6 to 63.7) median (range), in the control group to 103.5 μg/L (61 to 180.9) median (range), in the vaccinated group. Thereafter, there was no significant difference except on d 6 PV, when the SAA concentration was significantly higher in the vaccination group with a 2.8-fold ($P < 0.01$) increase from 42.5 μg/L (22.7 to 63.3) median (range) in the control group to 105.5 μg/L (63.8 to 238.1) median (range) in the vaccinated group (Figure 4).

DISCUSSION

This investigation showed that an intra-ocular vaccination of SPF layer chicks with a dual vaccine against Newcastle disease virus and infectious bronchitis causes an immediate but mild APP response, with small increases in AGP and SAA on d 2 and d 1 PV, respectively. We also demonstrated that the vaccinated birds underwent a mild vaccination stress response with H/L ratios remaining higher in the vaccinated birds for up to 3 d PV. The efficacy of the vaccine to produce antibody to the immunogens contained, was confirmed by demonstration of the presence of specific antibody on d 21 PV.

The SAA concentration in many species is related to the severity or virulence of the pathogen, and it can be used as marker for detection of inflammation (Eckersall, 1995; Ceron et al., 2005), serving as an indicator of the immediate innate immune response to stimulations such as vaccination. SAA usually increases within several hours (5 to 6 h in humans) of an inflammatory event and decreases after 48 h and typically increases 10- to 100-fold during a response (Kushner and Rzewnicki, 1994; Gruys et al., 2005). In the current study, the intraocular administration of N/B vaccine stimulated only a mild increase in the SAA concentration, though the serum SAA increased significantly at 24 h post treatment, with a 2.4-fold elevation, as is seen with vaccination in other species. For instance, SAA levels increased sharply from 5 h to hit the peak at 24 h to be 3.6-fold higher than the previous level before inoculation following intratracheal inoculation of beef calves with inactivated *Pasteurella multocida* (Dowling et al., 2004). In lambs, subcutaneous vaccination with

Heptavac P (combined *Clostridia* and *Pasteurella* vaccine) also caused a significant increase ($P < 0.01$) in both SAA and haptoglobin (**Hp**). The level of SAA peaked at 24 h, reaching a 400-fold increase, and did not return to normal concentration until 4 d later (Eckersall et al., 2008).

In the current study, we observed a second elevation of SAA in the vaccinated group compared to the control group on d 6 post treatment, which also coincided with a second peak in AGP concentration. A limitation of our study was that we were not able to repeat sample the same individual chicks, so it is not possible to determine if this second peak in APP was present because some individuals responded more slowly to the vaccination or if some other factor was coming into play, e.g., social stress. The most likely explanation is that the second APP peak was due to individual differences in the APP response (Verschuur et al., 2004; Elsasser et al., 2005).

In the current study, AGP in the vaccinated group was significantly higher by a 2-fold increase at 2 d post treatment. This result is similar to that reported by Sylte and Suarez (2012), who reported an AGP peak serum concentration of 2.6 -fold at 48 h post-experimental infection with influenza virus in 4 wk old White Leghorn chickens. However, we did not see a prolonged increase in AGP unlike the 96 h increase reported by these authors. Notably the intraocular inoculation with a vaccine strain of Gumboro disease virus lead to a 2.4-fold increase in plasma AGP at d 4 post treatment in 3 wk old white leghorn SPF chickens (Inoue et al., 1997) whereas intraocular inoculation with a highly virulent strain of Gumboro disease virus lead to an increase of plasma AGP at d 2 which peaked at d 6 post treatment (6.2-fold increase).

Measuring the H/L ratio has been established as means of evaluating stress in chickens (McFarlane and Curtis, 1989; Maxwell et al., 1992; Puvadolpirod and Thaxton, 2000; Post et al., 2003). In the current study, we observed an increase in heterophils and a decrease in lymphocytes in the vaccinated group, whereas the control animals did not show any significant change in their H/L ratios. The changes we observed in the H/L ratios was therefore likely to be related to the vaccine action and its effects on the innate immune response. These inflammatory mediators also initiate and modulate the APR, which by diffusing into the extracellular fluid and circulating in the blood, leads to the activation of the hypothalamic-pituitary-adrenal axis, decrease production of growth hormone and physiological changes as fever, lack of apatite and catabolism of muscle cells (Gruys et al., 2005). As a result, inflammation may have a combined effect on the H/L ratio by increasing heterophil concentrations due to increased granulocytopoiesis and decreasing lymphocyte concentration due to cytokine-mediated increases in corticosterone concentration (Clark, 2015). Change of the H/L ratio has been reported in birds to be 0.19 to 64.67 in different diseases (Clark, 2015), so for our vaccinated birds, a change in H/L from 0.11 to 0.54 is suggestive of a mild stress response.

Taken together, the vaccination procedure used in this study caused only a mild APR with only small increases in AGP and SAA, and a mild change in H/L ratios. This contrasts with results of PV acute phase reactions in other species and with other types of vaccine. In previous reports, the use of an adjuvant may have aided and enhanced the APR, e.g., in rabbit (Destexhe et al., 2013) and sheep (Eckersall et al., 2008). Thus, the absence of an adjuvant in the current N/B vaccine and administration by the intra-ocular route may have led to a moderate stimulation of the acute phase and stress responses.

In conclusion, vaccination of chicks with N/B vaccine by the intra-ocular route produced a mild acute phase response and vaccination stress response in 7-day-old SPF layer chicks. Of the 2 methods, the H/L ratio was more sensitive and consistent in terms of measuring the mild vaccination stress response under the conditions employed in this experiment. While the SAA and AGP levels increased within 1 and 2 d PV respectively, a limitation of the design of this experiment was that individual differences in the APR response could not be taken into account. For future work, it will be of interest to monitor the APR in the same individuals using different vaccines and routes of administration and to assess whether the post vaccine APPs response is correlated to both the H/L ratio and the subsequent antibody Titer. In addition, characterizing PV responses in chickens could be a way of assessing the dynamic APR and evaluating its potential as a biomarker for disease resistance.

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Quantitative proteomics using tandem mass tags in relation to the acute phase protein response in chicken challenged with *Escherichia coli* lipopolysaccharide endotoxin

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ABSTRACT

The inflammatory response in chickens (*Gallus Gallus domesticus*) is an integral part of the bird's response to infection. Detailing proteomic changes occurring during infection would be beneficial to the poultry industry, offering opportunities for comparative pathophysiological analysis. The objective of this study was to quantify the changes in the plasma proteome in chickens challenged with lipopolysaccharide (LPS), a bacterial endotoxin known to stimulate the host innate immune system. Plasma from chicken ($N = 6$) challenged with *Escherichia coli* (LPS) (2 mg/kg body weight) was collected pre (0 h) and at 12, 24, 48, and 72 h post-injection along with plasma from a control group ($N = 6$) challenged with sterile saline. Samples were analysed by a quantitative Tandem Mass Tags approach using a Q-Exactive-Plus mass-spectrometer. Identification and relative quantification were performed using Proteome Discoverer, and data were analysed using R. Gene Ontology terms were analysed by Cytoscape based on the *Gallus gallus* database. Finally, 87 significantly regulated proteins were found, including serum-amyloid-A, ovotransferrin and alpha-1-acid-glycoprotein, showing a significant effect of time post-injection in the LPS-treated group. Different pathways related with protein activation cascade and heterotopic cell-cell adhesion were affected by LPS-challenge. LPS-challenged chickens demonstrate significant changes to the plasma proteome with both increases and decreases of individual proteins within 12 h of challenge.

Significance: The injection of chicken with bacterial lipopolysaccharide followed by sequential plasma and clinical analysis of the bird, is a long established and a widely used model for inflammation and infection studies. This study, utilising and combining proteomic and immunoassay analysis with bioinformatic analysis, revealed that several biological pathways are modulated during this early period of inflammation. In addition, proteins with biomarker potential were identified and successfully validated. This experimental model also demonstrated potential for pathophysiological mechanism investigation and as an inflammatory model for biomedical research. There is, despite plasma being an easily accessible biological matrix which is representative of the health status of the bird, scarce data on the chicken plasma proteome. This research makes a positive contribution to the current field, generating significant data for continuing comparative analysis.

1. Introduction

Proteomics is now recognised as the principle method of investigating biological and pathological questions in veterinary medicine and farm animal production [1–3] and for uncovering biomarkers of infection and inflammation which, following validation, can be used both clinically and for livestock research. Furthermore, the use of proteomics in poultry research has also been initiated [4, 5]. Although more limited compared to other farm animal species, proteomics has been used to study a wide variety of issues in poultry sciences. Early studies focused on comparing different genetic lines that exhibit differing susceptibility to diseases such as coccidiosis [6] and Marek's disease [7]; or on comparing lines with differing growth and fat deposition characteristics [8]. The intestinal proteome is an apt target for proteomic studies in poultry with investigators studying the intestinal proteome during growth [9], in response to the bacteriocins [10], probiotics [11], high atmospheric ammonia exposure [12] and reused litter

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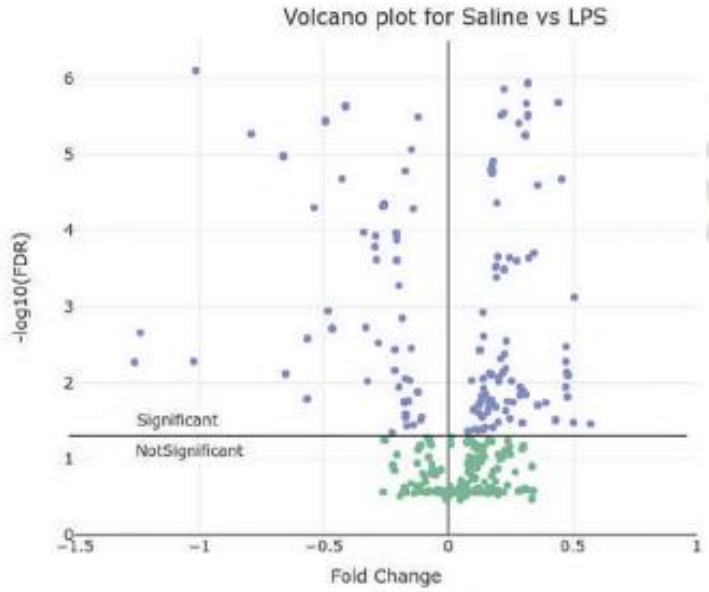

Fig. 1. Volcano plot for chicken challenged with *Escherichia coli* lipopolysaccharide endotoxin versus saline group.Volcano plot of fold changes (x-axis) and their associated log10 transformed p-values (y-axis) for the 571 peptides analysed by LC-MS. Peptides significantly different between saline and LPS groups ($\log10\ p > 1.3$) are in black, non-significant peptides ($\log10\ p < 1.3$) are in grey.

[13]. Metabolic diseases have also been investigated with the hepatic proteome of broilers afflicted with ascites syndrome compared to their normal counterparts [14] and the protein extracts of cartilage from normal and tibial dyschondroplasia (TD) affected growth plates compared [15]. The effects of heat stress on the hepatic proteome of chicken have also been detailed [16]. Proteomics is a research modality that readily applies itself to studies of poultry meat and egg quality with a number of studies investigating the proteome of muscle [17], carcass characteristics and meat quality [18]. The area of egg and egg shell research has utilised proteomic technologies with a significant number of proteomic studies detailing the egg and the egg shell/cuticle proteome [19–22]. Furthermore, serum proteomics of laying hens has been investigated in order to reveal the proteins playing the critical roles in egg production [23].

From an infectious disease perspective, the peripheral blood mononuclear cell proteome during Newcastle disease virus infection [24] and the proteomic changes induced in host cells infected with avian reovirus [25] have been characterised, as has brain tissue from chickens infected by the neurovirulent avian influenza virus [26].

Despite the variety and depth of such proteomic investigations, few studies have focused on the chicken's host response to infectious disease by investigating the plasma or serum proteome during an inflammatory response. A notable exemption has been the study of Packialakshmi et al. [27] demonstrating that, 24 h post treatment with *Salmonella typhimurium* lipopolysaccharide (LPS) endotoxin, proteins associated with immunomodulation, cytokine changes, and defense mechanisms were differentially abundant and as such may prove useful as biomarkers of infection and inflammation. The caecal proteome in response to *Salmonella enteritidis* infection has also been characterised [28]. Studies focused on femoral head necrosis (FHN) have found a number of differentially abundant proteins and potential biomarker candidates [27]. Investigations have led to spectral data from proteomic studies of chicken being placed in proteomic repositories. Accordingly, in Proteome Xchange/PRIDE repositories (available at https://www.ebi.ac.uk/pride/archive/) currently there are data related to about forty proteomic studies on *Gallus* species related to eggs, semen, uterus, tissue, and chromosomes, mostly produced by Orbitrap technology. Furthermore, in the PeptideAtlas and SRMAtlas repositories mass spectra derived from chicken liver, plasma, ovaries and oviduct have been deposited. As in other species integrating the current proteomic investigation with previously documented results from clinical and physiological analysis using methods such as immunoassay is a valuable exercise to cross-validate the approaches.

The injection of bacterial LPS followed by sequential plasma and clinical analysis of the treated birds, is long established and a widely used model for inflammation and infection studies with LPS from Salmonella or *Escherichia coli* being the most frequently used. Previous researchers have used this model extensively in studies of cytokine and acute phase protein (APP) the latter being plasma proteins known to change in concentration in response to infection or inflammation as part of innate immunity [29, 30]. APPs have been studied extensively across the veterinary species [31–33], including chickens [4] and the availability of validated immunoassays make their use for validation and comparative analysis especially useful. The major chicken APP is serum amyloid A (SAA), which can increase in plasma from 10 to 1000 fold [34], while alpha-1-acid glycoprotein (AGP) and ovotransferrin (OVT) are moderate APPs (3–10 fold increase) [35, 36] with ceruloplasmin (Cp) being a minor APP increasing 2–3 fold during an acute phase response [37, 38].

In order to investigate the immediate innate immune response of chicken to endotoxin induced inflammation LPS from *E. coli* was used to stimulate the response in broiler chickens. The objective of the study was to determine by relative quantification, using tandem mass tag (TMT) labelling and LC-MS/MS, the response of the plasma proteome, including that of APP, to endotoxin and to compare the proteomic re-

Table 1
Proteins with significantly differential abundance between LPS and saline groups identified using TMT approach.

Gene symbol (Gallus gallus)	Protein name	P-value (FDR)	Fold change
HPS5	Serum amyloid A protein	2.20E-03	1.24
SERPINA3	Alpha-1-antiproteinase *	7.89E-07	1.02
HPX	Haemopexin *	5.35E-06	0.79
ORM1	Alpha 1-acid glycoprotein	1.05E-05	0.66
LCN8	Extracellular fatty acid-binding protein precursor	7.62E-03	0.65
TF	Ovotransferrin	3.61E-06	0.49
CP	Ceruloplasmin *	1.14E-03	0.48
SMC4	Condensin complex subunit	1.94E-03	0.47
LOC107051143	Complement C3-like *	2.09E-05	0.43
CFD	Complement factor D, partial *	1.05E-04	0.34
LOC423629	Uncharacterized protein LOC423629 *	1.87E-03	0.33
LOC419851	Complement regulatory soluble protein	9.47E-03	0.32
PIT54	PIT 54	1.17E-04	0.29
LOC100858647	beta-Microseminoprotein-like *	2.99E-03	0.28
FGA	Fibrinogen alpha chain precursor	4.45E-05	0.26
CLU	Clusterin isoform X1 *	6.86E-03	0.21
FGB	Fibrinogen beta chain precursor	2.46E-04	0.21
FGG	Fibrinogen gamma chain precursor	1.10E-04	0.21
C3	Complement C3 precursor	1.41E-03	0.19
IGLL1	Immunoglobulin light chain variable region, partial	1.79E-02	0.18
CFHR2	Complement factor H *	1.64E-05	0.17
SPINK5	Ovoinhibitor	2.87E-02	0.17
A2ML4	alpha-2-macroglobulin-like protein 1 *	8.86E-03	0.17
VNN1	Pantetheinase precursor	3.69E-02	0.17
APOH	beta-2-glycoprotein 1 precursor	3.50E-03	0.15
FETUB	Fetuin-B precursor	8.55E-06	0.15
PLG	Plasminogen *	3.20E-06	0.12
ATRN	Attractin isoform X3 *	3.06E-02	0.11
LOC418892	Uncharacterized protein	2.78E-02	0.11
CST3	Cystatin precursor	2.25E-02	−0.10
TNC	Tenascin	4.09E-02	−0.11
ITIH3	Inter-alpha-trypsin inhibitor heavy chain H3 isoform X1 *	4.21E-02	−0.12
AGRN	Basement membrane-specific heparan sulfate proteoglycan core protein precursor	2.51E-02	−0.12
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2 precursor	1.96E-02	−0.12

Table 1 (Continued)

Gene symbol (Gallus gallus)	Protein name	P-value (FDR)	Fold change
IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit isoform X1 *	3.71E-03	−0.13
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein *	1.79E-02	−0.13
HRG	Histidine-rich glycoprotein *	1.51E-02	−0.14
COL5A1	alpha 1 (V) collagen	2.79E-02	−0.14
KNG1	Kininogen-1 *	1.19E-03	−0.14
C1QTNF3	Complement C1q tumor necrosis factor-related protein 3 isoform X1 *	8.85E-03	−0.14
F13A1	Coagulation factor XIII A chain	4.15E-02	−0.14
LUM	Lumican precursor	2.45E-03	−0.14
AHSG	alpha-2-HS-glycoprotein *	3.96E-02	−0.14
GIF	Hypothetical protein RCJMB04_7l4	3.80E-02	−0.15
PROS1	Vitamin K-dependent protein S *	1.42E-02	−0.15
APOA2	Apolipoprotein A-II *	2.47E-02	−0.16
ANPEP	Aminopeptidase, partial	2.11E-02	−0.16
LOC107056848	Cadherin-5-like, partial *	7.56E-03	−0.17
VTN	Vitronectin precursor	1.59E-05	−0.17
CL2	Ribonuclease CL2 precursor	1.69E-02	−0.17
LOC107055759	Vitamin K-dependent protein S-like *	7.86E-03	−0.17
APOA1	Apolipoprotein A-I	1.64E-05	−0.18
FBLN1	Fibulin-1, isoform D precursor	3.85E-02	−0.18
APOA4	Apolipoprotein A-IV precursor	4.10E-04	−0.19
IL6ST	Interleukin-6 receptor subunit beta precursor	4.32E-05	−0.20
TFRC	Chicken transferrin receptor	2.19E-04	−0.20
SPARC	Basement-membrane protein 40 precursor	3.29E-02	−0.20
F13B	Coagulation factor XIII B chain isoform X1 *	9.61E-03	−0.20
LOC100857892	Sushi, nidogen and EGF-like domain-containing protein 1 isoform X1 *	8.56E-03	−0.20
LOC107050076	IgGFc-binding protein-like, partial *	4.78E-03	−0.21
SERPINF1	Pigment epithelium-derived factor precursor	3.03E-06	−0.21
CLEC3B	Tetranectin precursor	7.32E-03	−0.22
SERPINC1	Antithrombin-III *	1.39E-06	−0.23
SERPINA4	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4 precursor	3.23E-04	−0.23
LOC771012	Coagulation factor X-like *	4.19E-03	−0.23
ENO1	alpha-Enolase	6.46E-03	−0.23
LOC100858068	IgGFc-binding protein-like, partial *	2.32E-02	−0.23

Table 1 (Continued)

Gene symbol (*Gallus gallus*)	Protein name	P-value (FDR)	Fold change
RBP4A	E Chain E, Retinol Binding Protein Complexed With Transthyretin	2.26E-04	−0.25
FN1	Fibronectin, partial	9.44E-03	−0.26
SERPINA5	alpha-1-Antitrypsin isoform X1 *	2.49E-04	−0.28
TGFBI	Transforming growth factor-beta-induced protein ig-h3 precursor	3.84E-06	−0.28
HBG2	beta-Globin	1.46E-02	−0.29
LCAT	Lecithin-cholesterol acyltransferase, partial	1.14E-02	−0.29
C4	Complement C4 precursor	3.37E-02	−0.30
ALPP	Intestinal-type alkaline phosphatase *	1.27E-02	−0.30
POSTN	Periostin precursor	3.16E-06	−0.32
COL1A1	Collagen alpha-1(I) chain *	2.52E-05	−0.36
SCARA5	Scavenger receptor class A member 5 isoform X1 *	1.97E-02	−0.36
LOC776376	Pentraxin-related precursor	1.82E-02	−0.39
HBAD	alpha-D globin	3.10E-02	−0.43
COL6A1	Collagen alpha-1(VI) chain precursor	2.07E-06	−0.44
CPN2	Carboxypeptidase N subunit 2 *	2.11E-05	−0.46
LRRC15	Uncharacterized protein LRRC15 *	2.11E-05	−0.46
HBAA	Hemoglobin subunit alpha-A chain	7.37E-03	−0.48
KRT8	Keratin, type II cytoskeletal 8 *	3.35E-02	−0.50
LOC107055417	Keratin, type II cytoskeletal 8-like, partial *	3.35E-02	−0.50
COL1A2	Collagen alpha-2(I) chain precursor	7.52E-04	−0.51

All proteins belong to the Gallus gallus proteome (UniprotKB).
* proteins predicted in Gallus gallus, with no evidence of existence to date at protein, transcript or homology levels.

sults to absolute quantification obtained by immunoassay of AGP, SAA and OVT, which are established APP in chicken plasma.

2. Material and methods

2.1. Experimental design

In March and April 2017 one day old, Ross 308 broiler chicks (PD Hook Hatcheries Ltd., Bampton, UK), were fitted with unique wing tags and housed in 4 groups of 14 in adjacent 1 m × 2 m pens on a litter of wood shavings on the University of Glasgow Cochno Farm & Research Centre. Broiler mash and water were available *ad libitum*. From the second day, one group per day was handled and moved into the trial room. All chickens were confirmed to be climatized to handling by 15 days old. Room temperature was maintained within the thermal neutral zone at 18 °C (range 18.0–18.3) and a 20 h:4 h light: dark cycle was implemented.

The experiment commenced when the chickens were 15 days old. Twenty four birds were injected subcutaneously (SC) at timepoint 0, with *Escherichia coli* lipopolysaccharide (LPS from *E. coli* O111:B4 purified by phenol extraction, L2630-25MG; Sigma-Aldrich, Dorset, UK) (2 mg/kg body weight) in a volume of 0.5 mL as the treatment group and another 24 birds injected SC by sterile normal saline (0.5 mL) as a control group. There were 5 blood sampling time points; pre (0 h) and post injection (PI) at 12, 24, 48, and 72 h. Plasma was collected from the same 6 chicken in the treated group and from the same 6 chicken in the untreated group at each time point for further analyses by proteomic and immunoassay methods. The remaining 18 birds in each group were not used in the plasma proteome investigation. Approximately 1.2 mL of blood was collected from the wing vein using heparinized tubes at each time point. The heparinised blood was centrifuged (3000 × *g*) for 15 min at 4 °C and the plasma aspirated and immediately frozen at −20 °C.

After the trial, all chickens were culled by over dose (1.5–2 mL/bird) i.v. injection of barbiturate (Euthatal 200 mg/mL, Merial, Woking, UK). Research was conducted under Home Office license (60/4466), and approved by ethical review of the University of Glasgow, MVLS College Ethics Committee.

2.2. Proteomic investigation of chicken plasma

Proteomic analysis of chicken plasma samples was performed by applying TMT-based quantitative gel-free approach as described previ-

Table 2
Proteins with significantly differential abundancee during time in chicken challenged with *Escherichia coli* lipopolysaccharide endotoxin.

Gene symbol	*P* value (FDR)	Fold change (12 h/0 h)	Fold change (24 h/0 h)	Fold change (48 h/0 h)	Fold change (72 h/0 h)
A2ML4	1.57E-02	0.34	0.44	0.31	0.19
AHSG	1.06E-02	−0.43	−0.15	−0.04	0.13
APOA2	1.37E-02	−0.36	−0.49	−0.19	−0.30
C3	4.39E-02	0.18	0.35	0.35	0.19
CFD	1.89E-02	0.42	0.61	0.62	0.43
COL1A1	3.39E-02	−0.62	−0.42	−0.42	−0.23
CP	3.16E-03	1.02	1.05	0.51	0.21
FGA	9.93E-03	0.29	0.38	0.23	0.03
FGB	1.03E-02	0.26	0.34	0.24	0.03
FGG	9.19E-03	0.23	0.33	0.23	0.01
HPS5	5.44E-03	2.27	1.35	0.35	−0.29
HPX	7.81E-03	0.76	1.20	1.21	0.94
ITIH2	4.46E-02	−0.19	−0.05	0.02	0.04
LCN8	1.82E-02	1.78	1.02	0.66	0.21
ORM1	5.01E-03	0.93	1.18	0.76	0.42
POSTN	2.16E-02	0.06	−0.30	−0.35	−0.15
SERPINA3	4.31E-03	1.24	1.35	0.99	0.73
TF	1.54E-02	0.51	0.55	0.41	0.30
TGFBI	1.50E-02	−0.34	−0.52	−0.29	−0.24

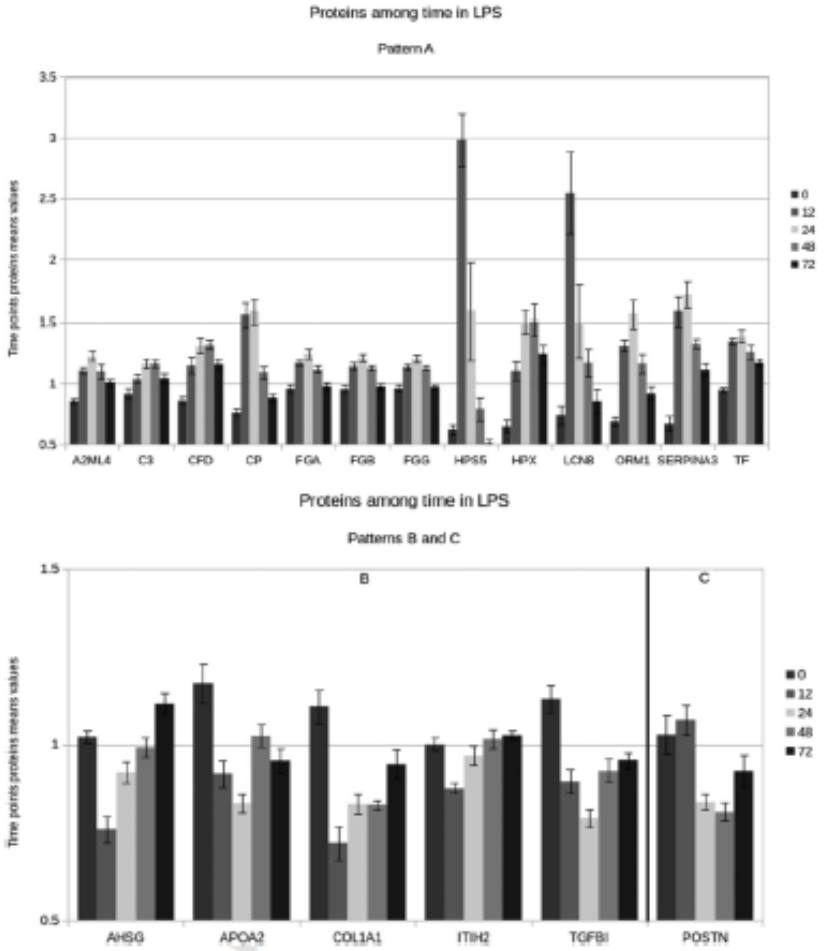

Fig. 2. Time-affected proteins in chicken challenged with Escherichia coli lipopolysaccharide endotoxin (LPS group).Barplot of the mean and SEM of 19 proteins differentially expressed for the different time points (0 h, 12 h, 24 h, 48 h, 72 h) in LPS group. Proteins have been grouped according to their pattern of expression: A or B and C. Patterns have been defined according to the evolution of fold changes among time.

ously [39]. In brief, after total protein concentration determination using BCA assay (Thermo Scientific, Rockford, USA), 35 µg of total plasma proteins from samples and internal standard (pool of all samples) were diluted to a volume of 50 µL using 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, USA), reduced by adding 2.5 µL of 200 mM DTT (60 min, 55 °C) (Sigma Aldrich, St. Louis, MO, USA), alkylated by adding 2.5 µL of 375 mM IAA (30 min, room temperature in the dark) (Sigma Aldrich, St. Lois, MO, USA) and acetone-precipitated (addition of 300 µL, overnight, −20 °C). Protein pellets were collected subsequently by centrifugation (8000 × g, 4 °C), dissolved in 50 µL of 0.1 M TEAB and digested using 1 µL of trypsin (1 mg/mL, Promega; trypsin-to-protein ratio 1:35, at 37 °C overnight). TMT sixplex reagents (Thermo Scientific, Rockford, IL, USA) were prepared according manufacturer's procedure and an amount of 19 µL of the appropriate TMT label was added to each sample used for the labelling reaction (60 min, room temperature) which was quenched using 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). Five TMT-modified peptide samples were combined with the internal standard (labelled with TMT *m/z* 126) into the new tube, aliquoted, dried and stored at −20 °C for further analysis. A total of 30 samples (6 chicken at 5 time points) from treated and 30 samples from control chicken led to 12 individual TMT experiments with the inclusion of internal standards in each experiment.

High resolution LC-MS/MS analysis of TMT-labelled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were loaded onto the trap column (C18 PepMap100, 5 µm, 100A, 300 µm × 5 mm), desalted for 12 min at the flow rate of 15 µL/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm × 75 µm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min, 45% to 90% for 2 min, held at 80% for 2 min and re-

Table 3
GO terms over-represented in chicken challenged with *Escherichia coli* lipopolysaccharide endotoxin *vs* saline group.

GOID	GOTerm	Term p-value	Genes	Cluster	Redundant	Leader	Group
GO:0072378	Blood coagulation, fibrin clot formation	1.18E-12	6	No specific			GO:0052547
GO:0031589	**cell-substrate adhesion**	**1.76E-04**	**7**	**No specific**		**Yes**	**GO:0031589**
GO:0015893	Drug transport	6.75E-03	4	Negative			GO:1902042
GO:0035987	Endodermal cell differentiation	5.95E-04	3	Negative			GO:0052547
GO:0030198	Extracellular matrix organization	6.46E-03	4	Negative			GO:0043062
GO:0043062	**Extracellular structure organization**	**8.25E-04**	**6**	**Negative**		**Yes**	**GO:0043062**
GO:0030195	Negative regulation of blood coagulation	1.75E-06	5	Positive	Yes		NA
GO:0010951	Negative regulation of endopeptidase activity	9.55E-18	16	Negative	Yes		NA
GO:2000352	Negative regulation of endothelial cell apoptotic process	4.89E-04	3	Positive			GO:1902042
GO:1902042	**Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors**	**4.17E-04**	**3**	**Positive**		**Yes**	**GO:1902042**
GO:0031639	Plasminogen activation	3.14E-04	3	Positive			GO:0052547
GO:0070527	Platelet aggregation	4.19E-03	3	Positive	Yes		NA
GO:0022409	Positive regulation of cell-cell adhesion	3.56E-03	4	Positive			GO:0031589
GO:0045921	Positive regulation of exocytosis	4.19E-03	3	Positive	Yes		NA
GO:0090277	Positive regulation of peptide hormone secretion	4.94E-04	4	Positive			GO:1902042
GO:0050714	Positive regulation of protein secretion	4.76E-03	4	Positive	Yes		NA
GO:0045907	Positive regulation of vasoconstriction	6.99E-04	3	Positive			GO:0052547
GO:0072376	Protein activation cascade	2.79E-13	9	No specific			GO:0052547
GO:0016485	Protein processing	1.58E-03	5	Positive	Yes		NA
GO:0030193	Regulation of blood coagulation	6.70E-05	7	No specific	Yes		NA
GO:0010810	Regulation of cell-substrate adhesion	9.15E-03	3	Negative	Yes		NA
GO:0051336	Regulation of hydrolase activity	1.52E-10	20	Negative			GO:0052547
GO:0052547	**Regulation of peptidase activity**	**1.62E-17**	**18**	**Negative**		**Yes**	**GO:0052547**
GO:0051592	Response to calcium ion	4.15E-03	3	Positive			GO:1902042
GO:0042060	Wound healing	7.79E-07	10	Negative	Yes		NA

GO terms were determined by Cytoscape/ClueGO and then analysed by REVIGO. GO terms in bold have been identified as the most representative of their GO group by the tool REVIGO.

equilibrated at 5% B for 20 min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water. Ionisation was achieved using nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 μm-inner diameter SilicaTip emitter (New Objective, USA). The MS operated in positive ion mode using DDA Top8 method. Full scan MS spectra were acquired in range from m/z 350.0 to m/z 1800.0 with a resolution of 70,000, 120 ms injection time, AGC target 1×10^6, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29% and 35% NCE) with a resolution of 17,500 and AGC target of 2×10^5. Precursor ions with unassigned charge state, as well as charge states of +1 and more than +7 were excluded from fragmentation.

For peptide identification and relative quantification the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., Thermo Fisher Scientific) was used. Database search against *Gallus gallus* FASTA files downloaded from NCBI database (7/12/2017, 46,105 entries) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. Only proteins with at least two unique peptides and 5% FDR were reported as reliable identification. Protein quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation The internal standard was used to compare relative quantification results for each protein between the experiments (sixplexes).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository [40] with the dataset identifier PXD009399.

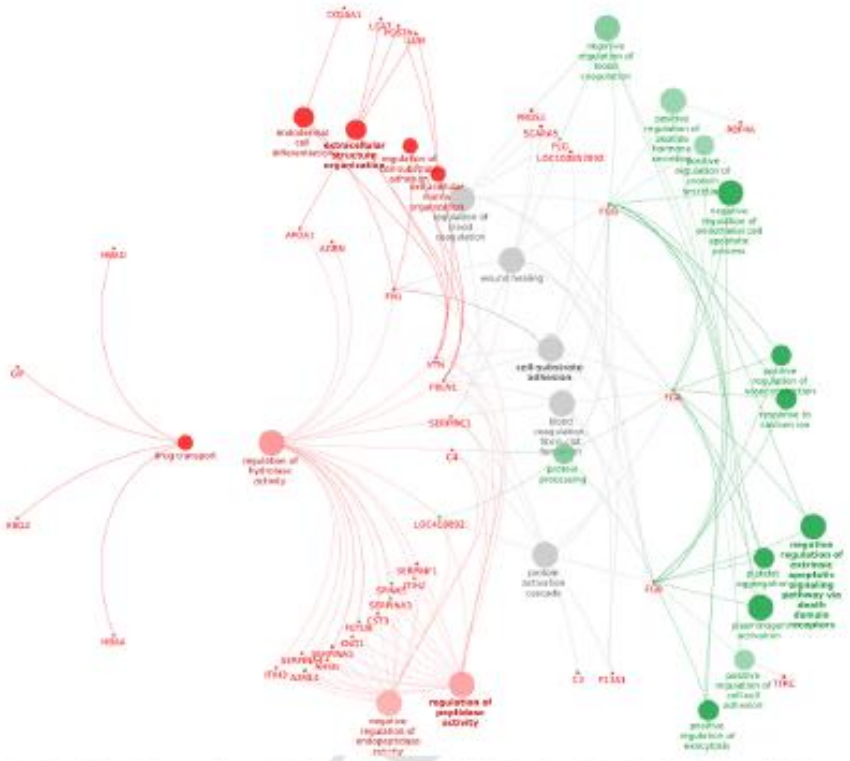

Fig. 3. Interactome of pathways differentially expressed between chicken challenged with *Escherichia coli* lipopolysaccharide endotoxin and saline, and their intermediate proteins.Gene ontology analysed pathways and proteins over-represented in LPS compared with saline samples. This analyse have been done with the Cytoscape application ClueGO and the REVIGO tool for GO terms selection. GO terms and proteins over-expressed in LPS are in green, lower-expressed in LPS are in red. GO terms in grey could not be attributed specifically to over or lower expressed terms/proteins. GO terms in bold represent GO terms selected to be the most representative of their GO group defined by the REVIGO tool. The yFiles radial layout algorithm was applied. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
GO terms over-represented in chicken challenged with *Escherichia coli* lipopolysaccharide endotoxin during time.

GOID	GOTerm	Term p-value	Genes	Redundant	Leader	Group
GO:0006953	Acute-phase response	7.12E-06	3			GO:0072376
GO:0010951	Negative regulation of endopeptidase activity	3.40E-05	4			GO:0072376
GO:0072376	**Protein activation cascade**	**6.11E-07**	**4**		**Yes**	**GO:0072376**
GO:0034116	**Positive regulation of heterotypic cell-cell adhesion**	**4.60E-07**	**3**		**Yes**	**GO:0034116**
GO:0051592	Response to calcium ion	5.96E-05	3			GO:0072376
GO:0070527	Platelet aggregation	5.32E-05	3	Yes		GO:0072376
GO:0045921	Positive regulation of exocytosis	5.32E-05	3	Yes		GO:0072376
GO:0042730	Fibrinolysis	3.15E-06	3			GO:0072376
GO:0045907	Positive regulation of vasoconstriction	7.12E-06	3			GO:0072376
GO:0050714	Positive regulation of protein secretion	1.72E-04	3	Yes		GO:0072376
GO:0090277	Positive regulation of peptide hormone secretion	4.96E-05	3			GO:0072376
GO:0031639	Plasminogen activation	3.15E-06	3			GO:0072376
GO:1902042	Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	3.99E-06	3			GO:0072376
GO:2000352	Negative regulation of endothelial cell apoptotic process	4.80E-06	3			GO:0072376

GO terms were determined by Cytoscape/ClueGO and then analysed by REVIGO. GO terms in bold have been identified as the most representative of their GO group by the tool REVIGO.

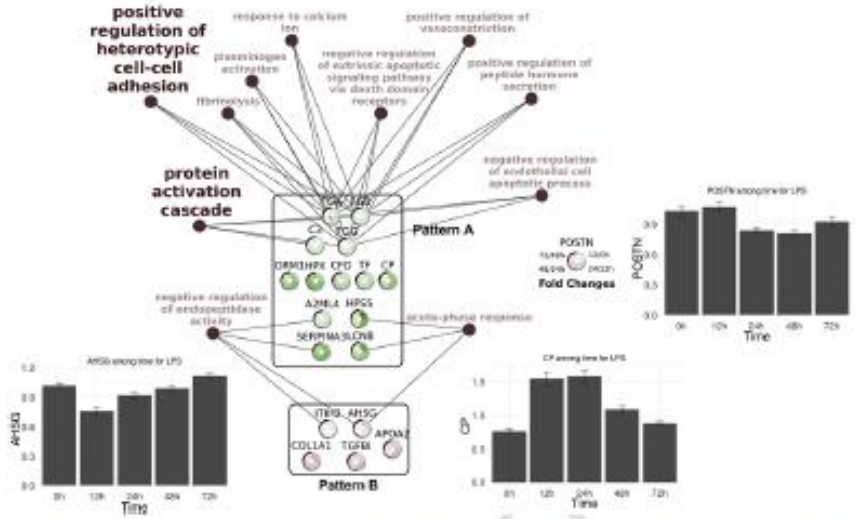

Fig. 4. Interactome of pathways differentially expressed among time in chicken challenged with *Escherichia coli* lipopolysaccharide (LPS group) and their intermediate proteins.Gene ontology analysed pathways over-represented in the list of 19 proteins differentially expressed among time in LPS group. This analyse have been performed with the Cytoscape application ClueGO and the REVIGO tool for GO terms selection. GO terms in bold represent GO terms selected to be the most representative of their GO group defined by the REVIGO tool. For each proteins, 4 fold changes among the 5 different time points have been represented using colour intensity to indicate fold change value. Positive fold changes are in green, negative are in red, fold change values close to 0 are in white. Proteins have been gather in 3 groups defined by their fold changes pattern. The A pattern corresponds to a rapid increase of a protein, then go back to the initial situation, while the pattern B corresponds to a rapid decrease of a protein and then a return to the initial situation. The C pattern corresponds to a decrease which happens later in the infection process. For each pattern, evolution of one protein mean among time has been represented with histogram to illustrate the pattern properties. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Group and time effects on alpha-1-acid glycoprotein (AGP), serum amyloid A (SAA), and ovotransferrin (OVT) proteins quantified by ELISA.

Protein	Group	Group × time	Time - LPS group	Time – saline group
AGP	< 0.001	< 0.001	< 0.001	NS
SAA	< 0.001	< 0.001	< 0.001	NS
OVT	< 0.001	< 0.001	< 0.001	NS

NS: Not Significant ($p > .05$). Group effect was assessed between LPS and saline samples by Wilcoxon-test (2-sided). Mixed Group × Time and Time effects were assessed by a Kruskal-Wallis test.

2.3. Statistical and bioinformatics analysis of the chicken plasma proteome

Statistics for proteomics: All statistics were performed using R (v3.4.3) [41] under the RStudio environment (v1.0.143) [42]. Infection effect (saline *versus* LPS) and time effect in infection groups (0h,12h,24h,48h,72h in saline and LPS separately) were considered for investigation. A peptide was not considered for the analysis if one of its group (infection, time or infection × time) had more of 50% of missing data after LC-MS identification and quantification.

A two-way ANOVA was performed to model the effect of treatment and time on the quantity of the peptides, using a linear regression model. Distribution of residuals generated by the ANOVA was accessed by a Shapiro-Wilk test. A Kruskal-Wallis test was performed to access the effect of treatment and time on peptides quantity using the R package "PMCMRplus" [43]. Due to multiple comparisons performed, a local False Discovery Rate was applied using the R package "qvalue" [44]. Each *p*-value was transformed by the function -log10(x).

Fold change between the 2 groups has been calculated by the function log2(Mean(Group2)/Mean(Group1)). A volcano plot was designed using the R package "plotly" [45]. Plots were generated with the "ggplot2" package [46]. Spearman's correlation was calculated to estimate the relationship between ELISA and LC-MS quantifications for the proteins AGP, SAA and OVT.

All operations were scripted in R to assure the automatization of the statistics pipeline to all peptides.

Bioinformatics: Proteins ID (*Gallus gallus*) were converted into Gene ID (*Gallus gallus*) by the platform DAVID (david-d.ncifcrf.gov/conversion.jsp) conversion tool. Gene Ontology enrichment analysis was performed using the Cytoscape (v3.6.0) [47] plugin ClueGO (v2.5.0) [48] on GO-Biological Processes (08/03/2018).

For treatment effect (LPS *versus* saline), two clusters of proteins differentially expressed between the 2 groups were set: one cluster for over-expressed proteins following LPS treatment, the other for proteins exhibiting lower-expression following LPS. The analysis was performed using the following parameters: evidence code = All, GO levels 3 to 15, minimal number of gene = 3, minimal percentage of gene = 3, Kappa score threshold = 0.4, *p*-values corrected by Bonferroni step down.

For time effect, differentially expressed proteins with time were analysed at once using the following parameters: evidence code = All, GO levels 3 to 8, minimal number of gene = 3, minimal percentage of gene = 3, Kappa score threshold = 0.4, p-values corrected by Bonferroni step down.

The two lists of GO terms over-expressed in the context of group and time effects were submitted to an analysis by REVIGO (revigo.irb.hr) [49] to remove redundant GO terms and group similar terms based

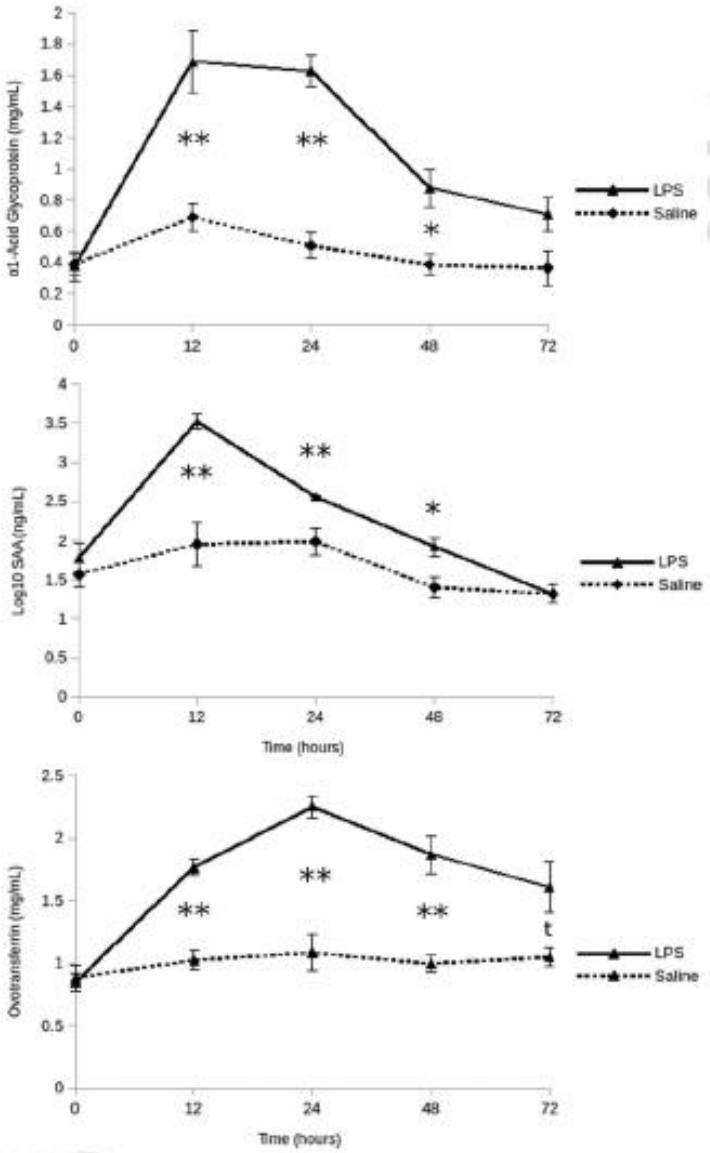

Fig. 5. ELISA quantification of alpha-1-acid glycoprotein (AGP), serum amyloid A (SAA), and ovotransferrin (OVT) in different time points.The quantity of each protein has represented in relation to the time points, for the 2 groups: LPS (continuous line) and saline (dash line). To better visualize differences in SAA, quantities have been transformed by the function log10 for the figure. SEM for each groups and time points have been added. Significance of differences between LPS and saline group for each time point separately have been represented. *$p < .05$, **$p < .01$ and t: $p > .05$.

on their description. For both analyses, the database used was *Gallus gallus*, with the SimReal semantic similarity measure.

Pathways of relationship between GO terms filtered according to REVIGO with their proteins/genes were designed on Cytoscape. Fold change data was included for the time effect analysis on samples from the LPS treated group.

2.4. Measurement of acute phase protein concentrations

Immunoassays: The concentrations of AGP, SAA and OVT were determined in the plasma according to previously described procedures [50]. The ELISA assays for chicken AGP and SAA were obtained from

Table 6
Time effect for alpha-1-acid glycoprotein (AGP), serum amyloid A (SAA), and ovotransferrin (OVT) proteins quantified by ELISA. A. Effect of group (LPS versus saline) for each time point, saline used as reference to compare. B. Effect of time on proteins fold change compared with 0 h (reference level).

A. Group effect (LPS versus saline) for each time point

		0h	12h	24h	48h	72h
AGP	Fold change	−0.04	1.29	1.68	1.18	0.96
	P value	NS	< 0.01	< 0.01	< 0.05	NS
SAA	Fold change	0.73	5.21	1.90	1.71	0.01
	P value	NS	< 0.01	< 0.01	< 0.05	NS
OVT	Fold change	−0.06	0.78	1.05	0.90	0.62
	P value	NS	< 0.01	< 0.01	< 0.01	< 0.1

B. Time effect in LPS group compared with 0 h

		12h/0h	24h/0h	48h/0h	72h/0h
AGP	Fold change	2.16	2.11	1.22	0.91
	P value	< 0.01	< 0.01	< 0.05	NS
SAA	Fold change	5.77	2.58	0.44	−1.53
	P value	< 0.01	< 0.01	NS	NS
OVT	Fold change	1.06	1.41	1.13	0.92
	P value	< 0.01	< 0.01	< 0.01	< 0.01

NS: Not Significant (p > .05). Differences were assessed with a Wilcoxon-test (2-sided).

Life Diagnostics Inc. (West Chester, USA). They were performed according to the manufacturer's instructions with a dilution factor for the plasma samples of 1:10000 for AGP and 1:20 for SAA. Each individual sample was run in duplicate. The plasma concentration of OVT was assessed by radial immunodiffusion (RID) using specific antibody for chickens OVT as described previously [51].

Statistics for immunoassays: Statistics on immunoassay were performed by non-parametric tests due to group size and distribution. Group effect was assessed by a Wilcoxon-test (2-sided), and a Kruskal-Wallis test was used to assess mixed effect Group × Time on all groups and Time effect on LPS and saline groups separately. For each time point (0 h/12 h/24 h/48 h/72 h), difference between LPS and saline was assessed by a Wilcoxon-test (2-sided) and fold change of expression calculated between times 12 h/24 h/48 h/72 h *versus* 0 h in LPS group. Correlation between these proteins was assessed on LPS group by a Spearman rank test.

3. Results

3.1. Proteomics

Protein identification and labelling efficiency: MS-based proteomic analysis is a powerful tool for high-throughput monitoring of quantitative plasma proteome changes. In this study, 418 quantifiable proteins (206 of which were master proteins and 95 master protein candidates, respectively) were identified according to set criteria (2 unique peptides and 5% FDR) [52]. To achieve the reliable and accurate quantification results, labelling efficiency (the percent of labelled peptides among all identified peptides) was examined by determination of TMT sixplex at peptide the N-terminus and K as dynamic modifications at the peptide level for all identified peptides [53]. By applying this approach, both labelled and non-labelled peptides can be simultaneously identified and obtained data used for labelling efficiency calculation. In the experiment, very low number of non-labelled peptides were identified indicating that TMT labeling efficiency was very high (about 98.5%).

Peptides distribution: From the initial list of 1243 quantifiable peptides detected by the LC-MS, 571 were considered after NA removals according to selection thresholds [52]. Residuals generated by ANOVA, were normally distributed for 264 peptides of the 571 analysed (46.2%), so a Kruskal-Wallis test was performed on all 571 peptides to study the effect of time and treatment on peptide quantities using the same approach.

Treatment and time effects: Treatment has a significant effect on 196 peptides illustrated on a volcano plot (Fig. 1). Among these, 43 showed a significant effect of time, but only in the LPS treated group. After using the DAVID conversion tool, a list of 87 proteins with significant differences between treatment and control was prepared (Table 1), and among these, 19 proteins had a significant time effect, but only for the LPS treated group (Table 2 and Fig. 2). Evolution of protein abundances among different time points by calculation of the relative change from 0 h (12 h *vs* 0 h, 24 h *vs* 0 h, 48 h *vs* 0 h, 72 h *vs* 0 h) revealed 3 distinct patterns. First, Pattern A characteristic of a rapid increase of protein followed by a progressive decrease to the initial, pre-LPS level exemplified by serum amyloid A (HPS5), and fatty acid binding protein (LCN8) which reached a peak at 12 h post treatment and also AGP (ORM1), ovotransferrin (TF) and haemopexin (HPX) which peaked at 24 h. Second, Pattern B, characteristic of a decrease of protein followed by a progressive increase to the initial level shown by alpha-2-HS-glycoprotein (AHSG) and apolipoprotein A-II (APOA2). Third, pattern C exhibited by periostin (POSTN) which had a small increase, then a decrease and finally an increase in protein quantity.

Gene Ontology analyses: Analysis of over-represented GO terms in treatment effect (saline *vs* LPS) generated a list of 25 GO terms (9 for lower-expressed following LPS treatment, 12 for over-expressed in the LPS treated group, and 4 which can not be attributed to over or lower-expressed groups) from 43 proteins of 87 (49.42%) considered after GO filtering application (Table 3). All those pathways have been represented with their shared proteins/genes (Fig. 3).

Analysis of over-represented GO terms in time effect of LPS (0 h/12 h/24 h/48 h/72 h) generated a list of 14 GO terms, 11 out of 19 proteins (57.89%) considered after GO filtering application (Table 4). All these pathways have been represented with their shared proteins/genes, enriched with fold changes data among time and grouped according to their pattern (A, B, C) (Fig. 4).

Acute phase protein concentrations: Plasma concentration of the proteins AGP, SAA and OVT were positively correlated. AGP was correlated with SAA and OVT ($r = 0.8$, $p < .001$ and $r = 0.54$, $p < .01$, respectively). SAA showed a tendency to be correlated with OVT ($r = 0.32$, $p < .1$).

Treatment (LPS compared with saline) had a significant effect on plasma concentrations of AGP, SAA and OVT proteins (p < .001 for the

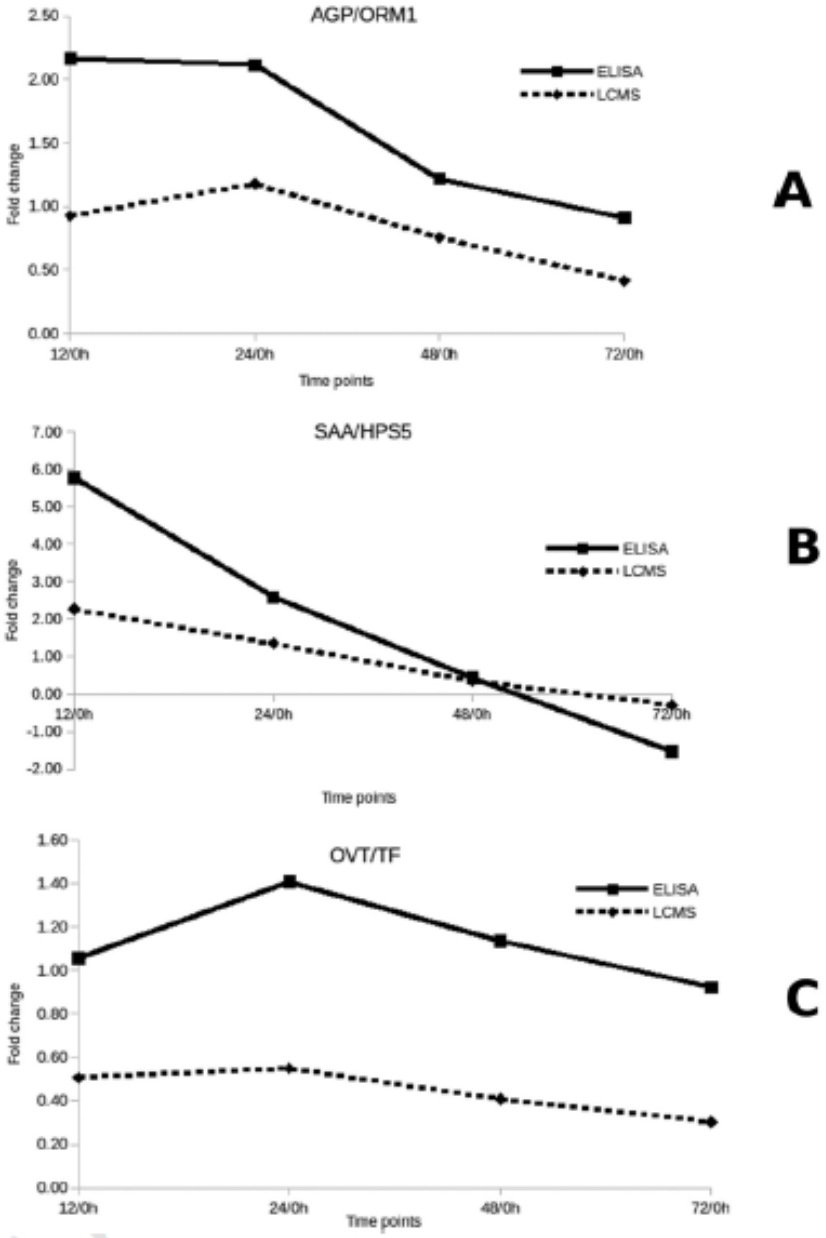

Fig. 6. Comparison of 4 fold changes among 5 time points performed by ELISA and LC-MS on alpha-1-acid glycoprotein (AGP), serum amyloid A (SAA), and ovotransferrin (OVT).Fold changes values have been represented for the 3 proteins to establish a comparison between ELISA and LC-MS quantifications: AGP/ORM1 (A), OVT/TF (B), and SAA/HPS5 (C).

three proteins) (Table 5, Fig. 5). There was a conjugated effect of group and time ($p < .001$ for the three proteins). Time has a significant effect on the LPS group ($p < .001$ for the three proteins), but not on the saline group ($p > .05$ for the three proteins).

The concentration of APP in the chicken plasma increased following LPS treatment. At 0 h (injection point) and 72 h (end of the experiment), levels of the three proteins are not statistically different between LPS and saline groups (Table 6A, Fig. 5) although for OVT, dif-

ference between 72 h and 0 h approached conventional level of significance ($p < .1$). At 12, 24 and 48 h, the concentrations of the three proteins were significantly higher in the LPS treated group compared to the saline treated control group (fold changes respectively for the three time points 1.29, 1.68, 1.18 for AGP; 5.21, 1.9, 1.71 for SAA; and 0.78, 1.05, 0.9 for OVT; with $p < .05$ for all).

In the LPS group (Table 6B, Fig. 5), there was a maximum peak of protein plasma concentration at 12 h for AGP and SAA (fold changes compared with 0 h at 2.16 and 5.77 respectively, with $p < .01$). The concentration compared with 0 h decreased with time (24 h, 48 h and 72 h) to be at 72 h at an undifferentiated level with 0 h time point ($p > .05$ for both AGP and SAA). For the plasma concentration of OVT (Table 6B, Fig. 5), the maximum level was reached at 24 h (fold change compared with 0 h at 1.41, $p < .01$) to decrease at 72 h to a level higher than 0 h (fold change 0.92, $p < .01$).

Comparison of immunoassay and LC-MS measurements: There were correlations between protein concentration measurements generated by ELISA and the relative abundance determined by LC-MS. For AGP/ORM1, the correlation coefficient was 0.74 ($p < .001$), for SAA/HPS5, the correlation coefficient was 0.58 ($p < .001$), and for OVT/TF correlation coefficient was 0.66 ($p < .001$). On converting the concentration of the APP determined by ELISA to fold change, the values were higher than the fold changes found by proteomics (Fig. 6).

4. Discussion

It is well known that different depletion strategies employed to remove high abundant plasma proteins, especially combined with multidimensional protein identification technology, lead to increased number of identified and/or quantified proteins [54]. In our study, non-depleted chicken serum was used for shotgun proteomic analysis. The main reason for using non-depleted plasma samples was a limited sample volume; insufficient for efficient and reproducible depletion using ProteoMiner which requires at least 100 μL of plasma according to manufacturer's procedure. In order to obtain the increasing number of proteins identified and quantified, we used optimized sample preparation (*e.g.* trypsin modified by reductive methylation, and thus resistant to proteolytic digestion) and LC conditions in terms of increased nanoLC column length and longer LC gradient time for peptide separation. In MALDI MS analyses, where peptide ions originating from trypsin autolysis can be used for internal calibration, it is desirable to use non-modified trypsin [55]. However, in shotgun proteomic analyses, products of trypsin autolysis can interfere with the LC-MS analysis depending on the trypsin-to-protein ratio used, as well as the extent of autolysis.

The use of TMT quantitative proteomics to follow the changes in the plasma proteome of chicken challenged with *E.coli* LPS endotoxin has revealed a time course of changes in high and low abundance plasma proteins yielding fresh insight into the chicken's innate immune response to LPS. This has revealed the extent of the fold changes experienced in both positive and negative directions of individual proteins, and identified the pathways most influenced following the LPS challenge. Chicken plasma proteins known to be stimulated positively during an acute phase response as part of the innate immune reaction to LPS, such as SAA, AGP, OVT and haemopexin were found to be increased in the plasma proteome, but in addition proteins such as alpha-1 antiproteinase and complement factor D were shown also to increase. Among the negatively responding proteins, apolipoprotein A2 is a recognised negative acute phase protein, being displaced in high density lipoprotein by SAA. Inter-α-trypsin inhibitor H2 (ITIH H2) was found to respond negatively, this is in contrast to other studies with species such as dog [56] and cow [57] in which it is recognised as a positive APP. Although the difference may be due to differences in the isoforms of this protein; it has been reported previously that ITIH H2 and H4 have been down- and upregulated, respectively, during an inflammatory response in human patients [58]. There has been a previous quantitative proteomics investigation of the chicken plasma proteome in response to endotoxin [27] but the response was only detailed at a single time point (25 h) after stimulation and with LPS from *Salmonella* Typhimurium. The changes in the plasma proteome described here in the response to LPS from *E. coli* are generally similar to those found with *S.* Typhimurium but there are also notable differences, which could be due to the contrasting LPS from the different bacteria, or to technical differences between the approaches taken. For instance, acetonitrile precipitation of high abundant proteins was used to pre-treat samples before MS by Packialakshmi et al. [27] but no pre-treatment was used in the investigation described here. Differences in the proteomes described include the identification of cathelicidin but not identification of SAA in the investigation of Packialakshmi et al. [27] which were the reverse of the findings here. There were however clear similarities in the protein changes such that the positive change in AGP and the negative response of APOA2 were found in both studies. It would be especially interesting if these changes were due to the nature of the LPS rather than from the methodological approaches used, as this could indicate that host responses to LPS differ between the type used in the challenge, but clarification on this would require further work.

This study has made further advances by determining the time course of the chicken plasma proteome response, with some protein fold changes reaching a maximum at 24 h (SAA) and others at 48 h (OVT). Such differences have the potential to be used in biomarker development for multiplex assays, either by targeted quantitative proteomics [59] or immunoassay [60]. Integration of a multiplex approach including proteins that have differing profiles of change, in positive and negative directions would be highly beneficial. In this challenge study, it is that both the positive and negative fold changes were of a temporary nature with return to pre-challenge values or were heading in that direction by 72 h post challenge. Statistically it was demonstrated that there was no effect of time on the saline control plasma samples but that there were significant difference with the LPS treated birds, demonstrating that neither the injection nor the associated handling had an impact on the plasma proteome, thus validating the saline group as controls. The finding that multiple proteins were elevated at the first time point 12 h after LPS challenge suggests that these, either singularly or in a combination could provide an early diagnostic indication that chicken are responding to a bacterial infection. Whether a biomarker panel could be developed on the basis of these findings would need further investigation and diagnostic validation, but this could be of marked value especially in the absence of clinical signs.

Pathway analysis showed that following infection, different pathways are activated (blood coagulation, exocytosis and protein excretion, vasoconstriction) and some are inhibited (apoptosis, extracellular matrix organization, peptidase/hydrolase regulation, drug transport). The fibrinogen complex, which has a role in blood coagulation cascade and immune response (both innate and T-cell mediated responses), is a critically important component of infection and inflammatory responses, it is also a potent proinflammatory mediator and as such a potential therapeutic target [61]. Fibrinogen, a noted positive APP in chickens as well as other species has been reported to be differentially abundant in chickens with *E. coli* and *Eimeria tenella* infection [37], as well as 24 h post induction by *Salmonella* Typhimurium LPS [27]. Other important actors are vitronectin (cell adhesion and blood coagulation), fibulin (blood coagulation, viral process), and antithrombin-III (blood coagulation, acute inflammatory response). Vitronectin is a glycoprotein promoting neutrophil infiltration enhancing the inflammatory process during infection, yet it binds to the bacterial surface protecting the gram negative bacteria from membrane attack complex-mediated lysis and enhances the adhesion of gram positive bacteria to

promote internalization [62]. Together with serpin A3–1, vitronectin-like protein was reported to be over-expressed during inflammation processes upon bacterial infection in cows [63]. Fibulin-1, an extracellular matrix and plasma glycoprotein, was also found to be up-regulated in serum of patients with bacterial meningitis [64].

During infection, some pathways show a specific variation pattern in time: vasoconstriction, apoptosis, plasminogen activation, protein activation cascade and acute phase response. Some of their key proteins have a pattern of difference among time following infection. There are 2 main groups of patterns: proteins which abundance raise quickly after infection (SAA, extracellular fatty acid-binding protein, alpha-1-antichymotrypsin and haemopexin for the most intense changes), and proteins which abundance decrease quickly after infection (apolipoprotein-A2, transforming growth factor 1, collagen alpha-1(I) chain for the most intense changes).

These results highlight how inflammation reaction occurs in response to a gram negative bacterial infection, through different cellular processes like vasoconstriction, protein excretion, cell movement and adhesion, apoptosis, blood coagulation, and key protein/complexes. Notable changes occurred 12 h after infection, with a number of pathways activated, and the host returned to a homeostatic state 72 h post LPS injection, as demonstrated by the absence of significant differences of plasma protein abundances between 72 and 0 h time points for most of proteins studied.

Measuring the APPs in the plasma provided quantitative data on their increased concentrations within 24 h of challenge and the subsequent return to pre-challenge levels. Comparison of fold changes between the immuno-based measurements and the fold change in AGP, SAA and OVT presents robust validation of the proteomic analysis and while only performed for these 3 proteins strengthens the wider findings and the fold changes in other proteins can be accepted as valid. One disparity between the immunoassay results and those from proteomics is that when the immunoassay results were converted to fold change these are, for all of the APP, higher than the fold changes found by proteomics (Fig. 6). This is likely to be due to the use of the standard in the TMT analysis composed of a pool of all samples, both from control and treated groups, while the fold increase in the immunoassay analysis is relative to the pre-treatment concentrations. The difference may also be related to the sensitivity of TMT-based LC-MS technology. Numerous studies have been conducted in order to compare the performance of immunoassays and LC-MS methodology, favoring the LC-MS in terms of accuracy and less false results. Nowadays, the approach by applying MS-based absolute quantification is often used in validation purposes, especially because of availability of (isotopically labelled) peptide standards and state-of-the-art instrumentation which provides high sensitivity and specificity circumvents the lack of specific antibodies, an issue especially problematic in veterinary research. It would be valuable to use the absolute quantification approach and standard peptides [55] to determine why such a difference in fold change from relative proteomics and from immunoassay results occurred in this study.

Establishing a quantitative proteomic method for assessing the relative plasma proteome response to LPS endotoxin in the chicken is potentially beneficial to researchers using the chicken as a model for early innate responses. It should also be of benefit to researchers from the poultry industry, for whom biomarkers for inflammation and infection provide the means to evaluate poultry health in a precise and quantitative fashion [65]. Following on, in a similar fashion to the early development of APP immunoassays, by establishing and validating this method in chickens, the endotoxin challenge model should be investigated alongside factors such as diet, breed, housing and vaccine efficacy. This will allow the measurement of plasma mediators for inflammation and infection to be fully elucidated to in these various and wide-ranging areas of poultry science.

5. Conclusions

A total of 87 proteins with significantly differential abundances between LPS and saline groups was found, and among these 19 proteins, including SAA, OVT and AGP, showed a significant effect of time post injection in the LPS treated group. Chickens challenged with bacterial endotoxin demonstrated marked changes to the plasma proteome with both increases and decreases found within 12 h of challenge. In conclusion, label-based high-resolution quantitative proteomic analysis and bioinformatics approach have been used to provide a valid tool for elucidating complex response of chicken plasma proteome challenged with LPS and uncovering relevant proteins with biomarker potential.

Conflicts of interest

The authors declare no conflicts of interest.

Uncited reference

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Appendix A. Supplementary data

Spectral data from the LC-MS/MS analysis has been uploaded to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD009399.

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Poultry red mite and responses of APP in laying hens. Manuscript have been accepted to publish in Poultry Science Journal at 21st of August 2018.

|Serum and acute phase protein changes in laying hens, infested with poultry red mite.

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ABSTRACT

The poultry red mite (PRM) is one of the most economically important ectoparasites of laying hens globally. This mite can have significant deleterious effects on its fowl host including distress, anaemia, reduced egg production and reduced egg quality. This study was conducted to evaluate the influence of PRM on the serum protein profile in laying hens and its effect on the acute phase proteins (APPs) to assess their potential as biomarkers for mite infestation.

Three acute phase proteins: alpha 1 acid glycoprotein (AGP), serum amyloid-A (SAA) and ceruloplasmin (CP) were measured in serum samples collected from laying hens at 12 and 17 weeks of age, and then for up to 4 months after a challenge with PRM (starting at18.5 weeks of age). The serum protein profile (SDS-PAGE /nanoflow HPLC electrospray tandem mass spectrometry) and concentration of individual serum proteins (SDS-PAGE-band densitometry) were also compared.

Post challenge there was a positive correlation ($r = 0.489$; $P<0.004$) between the levels of SAA and the PRM numbers. The levels of SAA steadily increased after the PRM challenge and were significantly different than the pre-challenge levels at 28, 32 and 36 weeks of age ($P< 0.01$). The PRM numbers also peaked around 31-33 weeks of age. The results for AGP and CP in comparison were inconsistent. Proteomics revealed the presence of two high molecular weight proteins in the serum between 12 and 17 weeks of age. These were identified as Apolipoprotein-B and Vitellogenin-2, and their increase was commensurate with the onset of lay. No other major differences were detected in the protein profiles of blood sera collected pre and post challenge. We conclude that SAA could be used as a useful biomarker to monitor PRM infestation in commercial poultry flocks and that PRM infestation does not

disrupt the production of the major proteins in the serum that are associated with egg formation.

Key Words: Acute Phase Proteins, Poultry Red Mite, *Dermanyssus gallinae*, Proteomics, Laying hen.

INTRODUCTION

One of the most important ectoparasites affecting the global poultry industry is the poultry red mite (PRM) (*Dermanyssus gallinae*) which is a haematophagous ectoparasite of domestic poultry and wild birds (Kristofik et al., 1996 ; Brannstrom et al., 2008). PRM reside off-host in the structure and furniture of poultry housing and emerge in darkness to feed on the hens every 2-4 days (Maurer et al., 1988). Serious health problems appeared when the PRM infestation level reaches 150 000 to 200 000 mites per bird (Kilpinen, 2005). PRM causes a significant deleterious effect on its avian host such as a high level of psychogenic stress (Kowalski and Sokol, 2009), decrease in egg production, anaemia, blood staining of eggs and an increase in mortality rate (Chauve, 1998; Kilpinen, 2005). Traditionally, PRM control often relies upon synthetic acaricide spraying of poultry houses and equipment; however, resistance against these compounds has been reported to limit efficacy (Beugnet et al., 1997; Sparagano et al., 2014). The number of acaricides licenced for PRM treatment is severely limited in the European Union (Flochlay et al., 2017) but there is evidence of illegal use of banned acaricides (Marangi et al., 2012), including detection of Fipronil residues in eggs from laying flocks in several EU countries (Anonymous, 2017).

The use of acute phase proteins (APPs), as physiological markers for assessment of overall health and welfare has potential for diagnosis and prognosis in veterinary medicine (Ceron et al., 2005; Cray et al., 2009; Ceciliani et al., 2012), thus allowing the more rational and targeted use of drugs and treatments. Acute phase proteins are classified into positive and negative according to their response increasing or decreasing after stimulation respectively (Chamanza et al., 1999). The positive type can be sub-divided into major, moderate and minor protein classes depending on their response magnitude (Ceron et al., 2005). Alpha 1-acid glycoprotein (AGP) is a moderate positive APP in most species. The main function of AGP is to act as a natural anti-inflammatory factor inhibiting neutrophil activation and

55 increasing the secretion of IL-1 receptor antagonists by macrophages (Murata et al., 2004).
56 This has a clearance function in removing bacterial lipopolysaccharide (LPS) by binding and
57 neutralizing its toxicity (Murata et al., 2004) and it also acts as a plasma transport protein
58 (Ceciliani and Pocacqua, 2007). AGP is one of the most widely measured APPs in chickens
59 owing to the commercial availability of easy to use and chicken-specific ELISA kits
60 (O'Reilly and Eckersall, 2014; Peebles et al., 2014; Koppenol et al., 2015). Another APP
61 with known beneficial biological functions is Ceruloplasmin (CP). Like AGP, CP is a
62 moderate positive APP in chickens (Georgieva et al., 2010). The main function of CP is to
63 transfer and store copper within the body, and it also has anti-oxidative functions (Floris et
64 al., 2000). *In vivo* studies have shown that CP has the ability to oxidise the Fe^{2+} ion,
65 preventing the iron inducing oxidation effect (Floris et al., 2000). Serum amyloid-A (SAA) is
66 a major positive APP in chickens (Alasonyalilar et al., 2006). The main function of SAA is to
67 modify the transport and metabolism of lipoprotein in the acute phase response (APR)
68 including, specifically, cholesterol transport to damaged tissues. This is essential for repair
69 and reconstruction of cell membranes and to clear lipid debris formed by bacteria and
70 damaged tissues (O'Reilly and Eckersall, 2014). SAA also has a modulatory role to inhibit
71 pyrexia and pro-inflammatory events during the APR (Shainkin-kestenbaum et al., 1991;
72 Uhlar and Whitehead, 1999). In this study we investigated if the levels of AGP, CP and SAA
73 change in response to a PRM infestation in laying hens.

74 In addition to APPs, there has been growing interest in the use of advanced proteomic
75 analysis to assess changes in the serum proteome of poultry and other livestock in order to
76 identify additional biomarkers of disease (Almeida et al., 2015). In commercial layers the
77 onset of lay requires a major change in the physiology of the hen, with nutrients such as lipid
78 and protein being required in copious amounts for egg production. With the exception of
79 immunoglobins, synthesis of most of the major egg yolk proteins (Vitellogenins and VLDLs)

takes place in the liver predominantly under the regulation of estrogen and to a lesser extent androgens. These egg yolk precursors are then transported in the blood to the ovary where they are further processed to phosvitin, lipovitellin, triglycerides, cholesterol, and phospholipids within the developing oocyte (Johnston 2015). To date there have been few investigations of the alteration this causes in the serum proteome of hens as they approach sexual maturity. Libu et al.(2007) however did report that Vitellogenin and Apolipoprotein-A1 changed dramatically at peak egg production relative to initial egg production and went on to demonstrate an association between these proteins and egg production in different groups of Taiwan red-feathered country chickens. In the current study we wanted to evaluate if a PRM challenge disrupts the transport of these and other major proteins in the blood serum required for egg production.

The aims of this study were therefore i) to investigate the APP response over the time course of an experimental PRM infestation in laying hens ii) to demonstrate the major changes in serum proteome following the onset of lay and iii) to evaluate if a PRM infestation significantly alters the serum protein profile of laying hens.

MATERIALS AND METHODS

Treatment and Sampling

This study was performed using additional data collected from the control hens which were part of a vaccine field trial carried out by the Moredun Research Institute (Bartley et al., 2017). The hens were Lohmann Brown Hens (n=384) and as the 'controls', each hen had received two injections of a 0.5 ml dose of a vaccine placebo of Montanide ISA70VG adjuvant (SEPPIC, Terrassa Bellini, Paris) formulated in 7:3 ratio with 10 mM Tris–HCl; 0.5 M NaCl, pH 7.4 at both 12 and 17 weeks of age. At the time of the 2nd placebo vaccine injection (week 17) the birds were placed in cages, with four birds per cage. At 18.5 weeks of

age, all 96 cages were subsequently infested with PRM (10,000 live mites per cage). This infestation rate was used so that the experimental cages were not immediately overwhelmed with mites (Bartley et al., 2017). Every two weeks for four and a half months post challenge (Figure 1) the PRM populations were estimated using a trapping and counting procedure (Bartley et al., 2017). Plastic ADAS Mite Monitor traps (ADAS Ltd., Oxon, UK) were fixed to the egg collection tray at the front of each cage. After 24 h the mites in the traps were collected into individually-marked plastic containers containing 70% (v/v) ethanol and counted.

Blood samples were collected at a) placebo vaccination 1 (pre-PRM challenge, week 12); b) placebo vaccination 2 (pre-PRM challenge, week 17); c) 5 days post -PRM challenge (week 19); d) at times which coincided with alternate PRM trap evaluations viz. Weeks 23, 27, 31, 36 and 38 weeks post -PRM challenge (Figure 1). At each sampling time point, blood was collected from 8 randomly selected birds by bleeding directly from the wing vein into a non-heparinised tube. The blood (1ml) was then allowed to clot at 4 °C for 24 h and the sera were obtained following centrifugation at 3000 g for 10 minutes. Sera were stored at -20°C, until required for further analysis.

The PRM experiment was performed under the terms and conditions of UK Home Office licences (PPL 60/ 4324) and the experimental design was ratified by the ethics committee of the Moredun Research Institute.

ELISA Assay

The chicken specific ELISA assays for AGP, SAA and CP were obtained from Life Diagnostics Inc, (West Chester, USA). Each assay was performed according to the manufacturer's instructions using conditions previously validated (Kaab et al., 2017). Serum

was diluted 1:10000 for AGP, 1:20 for SAA and 1:40000 for CP. Each individual serum sample (n=8) from each sampling point (n=8) was analysed in duplicate.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For each of the 8 sampling times equal aliquots of sera (n=8) were pooled for SDS-PAGE using the method described in Thomas et al., (2016).

Nanoflow HPLC Electrospray Tandem Mass Spectrometry (nLC-ESI-MS/MS)

Eleven selected protein bands were excised manually by scalpel and placed in individual vials to be subjected to in-gel digestion for protein extraction prior to identification via mass spectrometry analysis. Gel pieces were washed with 100 mM ammonium bicarbonate for 30 minutes and then for a further hour with 100 mM ammonium bicarbonate in 50% (v/v) acetonitrile. After each wash all solvent was discarded. Gel plugs were then dehydrated with 100% acetonitrile for 10 minutes prior to solvent being removed and dried completely by vacuum centrifugation. Dry gel pieces were then rehydrated with 10µl trypsin at a concentration of 20ng/µl in 25mM ammonium bicarbonate (Cat No. V5111, Promega, Madison, WI, USA) and proteins allowed to digest overnight at 37°C. This liquid was transferred to a fresh tube (first extract), and gel pieces washed for 10 min with 10µl of 50% acetonitrile. This wash was pooled with the first extract, and the tryptic peptides dried.

Proteins were identified using nanoflow HPLC electrospray tandem mass spectrometry (nLC-ESI.MS/MS) at Glasgow Polyomics. Peptides were solubilized in 2% acetonitrile with 0.1 % trifluroacetic acid and fractionated on a nanoflow uHPLC system (Thermo Scientific RSLCnano) before analysis by electrospray ionisation (Collgros, et al. 2013) mass spectrometry on an Amazon Speed ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (Thermo Scientific). Peptides were desalted and concentrated for 4 min on a C18 trap column followed by an acetonitrile

gradient (in 0.1% v/v formic acid) (3.2 - 32% v/v 4 - 27 min, 32 % to 80% v/v 27 - 36 min, held at 80 % v/v 36- 41 min and re-equilibrated at 3.2 %) 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 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 and with the FDR set at 0.01.

MS data were processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server. Protein identifications were assigned using the Mascot search engine to interrogate protein sequences in the NCBI Genbank database, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses and with the Mascot score set off as 100.

Protein Band Densitometry Measurements

To determine the protein concentration in each electrophoretic band of interest and to account for individual bird variation, serum samples from four individual birds at each time point were run on 1D SDS-PAGE gels. The concentration of each band was then estimated by protein band densitometry using ImageJ software (https://imagej.nih.gov/ij/), and expressed as a mean percentage of the total lane protein. The mean percentage for each sample (n=4 birds) were then converted to mg/ml by calculating the quantity of the band of interest as a proportion of the density of the whole lane, where 100% equals 2mg/ml (the original concentration of the loaded samples).

Statistical Analysis

A Mann-Whitney Test (Minitab 17.1.0) was used to examine the effect of PRM infestation on each APP by comparing serum samples taken prior to the PRM challenge (weeks 12 and 17) with those taken following the challenge (weeks 19, 23, 27, 31, 36, and 38).

Spearman's correlation was used to determine the relationship between levels of PRM infestation on specific APPs levels (GraphPad Prism v.5 software). All statements of significance are based on testing at $P \leq 0.05$.

RESULTS

Poultry Red Mite (PRM) Infestation Levels

The mean number of PRM per trap is presented in Figure 2. Low numbers of PRM were recorded in the first traps at week 21 and 23. Following this initial lag period the PRM population expanded and the number of PRMs caught in the traps increased sharply and peaked in weeks 31-33 (Figure 2). Following the peak, the PRM numbers decreased in weeks 36 (late August) and 38 (September).

Serum Concentration of Acute Phase Proteins and Their Relationship to PRM Infestation Rates

Serum Amyloid-A (SAA). There was a significant increase in the concentration of SAA in serum obtained post infestation at 27 ($P<0.05$), 31 and 36 weeks ($P< 0.01$) compared with pre-infestation (weeks 12 and 17) levels (Figure 3a). The pattern of change in the mean SAA levels closely mirrored the mean PRM levels of infestation (Figure 2). To investigate this relationship further, data where the PRM infestation levels could be directly matched to a blood sample from the same cage were identified and analysed using Spearman's correlation. This revealed a moderate positive correlation ($r = 0.489$; $P < 0.004$) between SAA levels and the numbers of PRM.

Alpha-1 Acid Glycoprotein (AGP). There was no consistent change in the serum AGP levels pre and post PRM challenge. Levels of serum AGP prior to infestation (week 12) were significantly higher than at week 19 ($P<0.05$) and 27 ($P< 0.01$). AGP levels following the PRM challenge were not significantly different than that observed at 17 weeks, Figure 3b. In addition, there was no correlation between the AGP concentration and PRM infestation levels ($r = 0.257$; $P < 0.6$).

Ceruloplasmin (CP). Serum concentrations of CP at 12 weeks were significantly higher ($P< 0.05$) than at most time points post-infestation, with the exception of week 17 (also a pre-challenge time point) (Figure 3c). CP levels were not correlated with PRM infestation level ($r = 0.328$; $P < 0.3$).

SDS PAGE and Identification of the Major Serum Protein Components

SDS PAGE separation of the pooled serum samples from all sampling points are compared in Figure 4 and the top three proteins in terms of MOWSE score for each band are given in Table 1 with full details of the proteomic analysis provided in the supplementary data. Two high molecular weight (MWt) bands of approximately 300 kDa (band 1) and 250 kDa (band 2) were absent in the 12 week sample but present in all of the other samples. The 300 kDa protein (band 1) corresponded to *Gallus gallus* Apolipoprotein-B precursor (gil113206052) with coverage 40%. The 250kDa protein (band 2) corresponded to *Gallus gallus* Vitellogenin-2 (gil71896765) with coverage of 66%. Bands 3 and 5 with MWt of ~200 and 100 kDa both corresponded to the same protein (Alpha-2 macroglobulin like protein). Ovotransferrin, Albumin, Apolipoprotein A-IV and Apolipoprotein A-I were amongst the other proteins identified.

Protein Densitometry Measurements

Figure 5 compares the relative concentration of proteins in band 1-11 between 12 and 17 weeks of age for four individual hens selected at random at each time point. As well as there being a significant increase in Apolipoprotein-B (MWt ~300 kDa; P = 0.007) and Vitellogenin-2 (MWt 250 kDa; P = 0.007) there was a significant decrease in Alpha-2 macroglobulin's (~200 kDa, band number 3; P = 0.007 and ~100 kDa, band number 5; P = 0.007) and Apolipoprotein A-1 (28 kDa; P = 0.003) between 12 and 17 weeks of age.

Protein densitometry measurements carried out on serum from four individual hens at each sampling time point pre and post PRM challenge revelled that Apolipoprotein-B levels increased 22.6-fold (from 0.008 mg/ml to 0.181 mg/ml; $P \leq 0.01$) and Vitellogenin-2 increased 100-fold (from 0.002 mg/ml to 0.20 mg/ml; $P \leq 0.01$) between 12 and 23 weeks of age and remained high throughout the study period (Table 2). Meanwhile Apolipoprotein A-I (band 11) decreased by 2.1-fold (from 0.193mg/ml to 0.09 mg/ml, $P < 0.05$) at 23 weeks compared to week 12. Other protein bands were also compared but no statistically significant differences were observed other than that for Alpha-2 macroglobulin (band 3 and 5) between 12 and 17 weeks, as previously reported (data not presented).

DISCUSSION

Infestation of laying hens with PRM resulted in an APP response which was characterised by SAA concentrations being 6.25-fold higher in the blood sera at 31 and 36 weeks compared to week 17 pre-infestation levels ($P<0.01$). Mite infestation has been reported to cause a significant increase in SAA in other species. For example, experimental infestation of sheep with the sheep scab mite (*Psoroptes ovis*) led to a significant elevation of SAA ($P \leq 0.001$) compared to pre-infestation levels (Wells et al., 2013). A similar SAA response has also been reported in the case of mange infestation (*Sarcoptes scabiei*) in the Alpine ibex (Rahman et al., 2010), with an SAA peak occurring at week 4-5 post infestation. The serum concentration

243 of SAA in the current study was moderately correlated to the PRM infestation levels (r =
244 0.489; P < 0.004), suggesting that exposure to PRM may trigger an APP response probably
245 mediated by cytokines released by the cells of the innate immune system in response to the
246 mites feeding (Kowalski and Sokol, 2009). However, less than 50% of the variation in PRM
247 can be predicted from the relationship between SAA and PRM. This is not unexpected as
248 there are many other factors which can affect the numbers of PRM including the
249 environmental conditions (Sparagano, et al., 2014), the feeding behaviour of the mites and
250 the acquired as well as innate immune responses of the host. APPs can also respond to
251 increased corticosterone (Zulkifli et al., 2014), which has previously been shown to rise in
252 response to PRM infestation in laying hens (Kowalski et al., 2006). Monitoring SAA levels
253 could therefore be valuable as an aid to assessing the PRM infestation level and the welfare
254 of laying hens in poultry farms and as a means to monitor the innate immune response
255 mediated via pro-inflammatory cytokines (Baumann and Gauldie, 1994, Jensen and
256 Whitehead 1998; O'Reilly and Eckersall 2014)

257 Neither AGP nor CP levels increased in response to the PRM infestation. The pro-
258 inflammatory cytokine stimulation of the acute phase response was therefore relatively
259 moderate as it did not stimulate the production of these two APPs. In other species, SAA is
260 one of the most sensitive major APPs to stimulation (Ceron et al., 2005; Ceciliani et al.,
261 2012). Consequently, a similar immunological trend might be true for laying hens. In future
262 studies it would be useful to monitor the changes in pro-inflammatory cytokines such as
263 interleukin 1 and interleukin 6, to confirm that only a moderate cytokine response had been
264 stimulated.

265 The serum concentrations of AGP and CP fell in the early stages of our study (Figure 3).
266 Such subtle changes may reflect an age-related alteration in these two APPs; age related
267 differences in APPs have been reported in other species such as in pig (Christoffersen et al.,

2015), but until now have not been reported in laying hens. The fall in CP between 12 and 19 weeks of age for example may be associated with the onset of sexual maturity and oogenesis. Oocyte development requires the presence of various elements, including copper that binds specifically to egg proteins such as Ovotransferrin (Johnson, 2015). CP contains over 95% of the copper found in the plasma (Martínez-Subiela et al., 2007). The high demand for copper associated follicle development at the onset of lay therefore provides a plausible explanation for the observed reduction in serum CP levels in our study.

Additional changes in the serum proteome were investigated in 4 individual samples from each time point using SDS-PAGE gel electrophoresis and protein densitometry. Both Apolipoprotein-B (MWt 300 kDa) and Vitellogenin-2 (MWt 250 kDa) were significantly increased by 17wks and reached a peak by 23wks which is just in advance of peak production for Lohmann brown layers. These results were obtained by densitometry of serum electrophoresis, a standard procedure in veterinary diagnostic laboratories (Eckersall, 2008), but which is not specific and it would be of value in future investigation to apply quantitative methods of greater specificity such as immunoassay or quantitative proteomics. However the results obtained here indicate the large increases in Apolipoprotein-B and Vitellogen-2 at week 17 and also the time of their maximum level in chicken serum. Both Apolipoprotein-B and Vitellogen-2 are lipid-binding proteins, and are precursors of the major proteins of the yolk (Finn, 2007). At sexual maturation, and in response to rising levels of oestrogen, plasma levels of Vitellogenin have been shown to rise as production in the liver increases (Wallace, 1985; Johnson, 2015). In the ovary, Vitellogenin is taken up by the developing oocytes to form two lipovitellins, phosvitin by proteolytic cleavage mediated by Cathepsin D (Bourin et al., 2012). In addition, Vitellogenin (and albumin) is important in the transport of circulating non-diffusible protein bound calcium, which is important for eggshell formation (Johnson, 2015). Liou et al. (2007) reported that Vitellogenin levels in the plasma were positively

293 correlated to egg productivity in a local breed of chicken in Taiwan (TRFCC). It would be of
294 interest to determine if a similar relationship exists in more highly selected breeds of layer
295 chickens.

296 Oogenesis is also considered to be the main factor affecting the plasma levels of
297 apolipoproteins and total lipid in chicken plasma, thus enabling efficient delivery of very
298 low-density lipoproteins (VLDL) to the egg (Pinchasov et al., 1994). Apolipoprotein B is a
299 major protein of VLDL. This protein undergoes proteolytic cleavage in the yolk into at least
300 nine protein fragments, the presence of some of which have been shown to contribute to the
301 excellent emulsifying properties of egg yolk (Jolivet et al., 2008). Apolipoprotein A-I is
302 involved in incorporating lipid into the yolk mass (Vieira et al., 1995), so the demand for this
303 protein will also be greatest at peak production (Finn, 2007). Interestingly Liou et al. (2007)
304 found that the levels of Apolipoprotein A-I were negatively correlated to egg productivity in
305 their study of a native Taiwan breed of chicken. Alpha-2 macroglobulin (bands 3 and 4,
306 Figure 5) significantly decreased between 12 and 17 weeks and then remained at a relatively
307 constant level for the remainder of the experiment. This protein is a part of the innate immune
308 system and functions to clear active proteases from tissue fluids (Armstrong and Quigley,
309 1999). Alpha-2 macroglobulin also known as Ovostatin has been isolated from the plasma of
310 vertebrates as well as the egg white of birds and reptiles (Armstrong and Quigley, 1999;
311 Rehman et al., 2013). A decrease in plasma levels of this protein at the onset of lay may be
312 related to oviduct development and the increased demand for this protein to be incorporated
313 into the forming egg. Previous studies on human plasma samples have reported that the MWt
314 of Alpha-2 macroglobulin is 180 kDa and 85kDa (Harpel and Brower, 1983). In the current
315 study, the MWts were ~200 and ~100 kDa (Figure 4). This could be related to differences in
316 the degree of glycosylation, species variance, or methodology differences.

317 In relation to the third aim of this study, which was to determine if PRM significantly alters
318 the serum protein profile of laying hens, we did not find any differences using SDS-PAGE
319 which could be directly attributable to the PRM challenge (Figure 5). Changes in SAA were
320 only observed using an ELISA which had the sensitivity to measure this low abundance
321 protein. SAA has a low molecular weight (14kDa) when it becomes dissociated from the high
322 density lipoprotein and is known to be a difficult protein to analyse using SDS-PAGE (Soler
323 et al., 2013). This, together with SAA having a concentration in the µg/L range (>1,000 lower
324 than high abundance serum proteins such as albumin and IgG which have concentrations of
325 1-50 g/L) means that electrophoresis is not suitable for monitoring this acute phase and low
326 abundance protein.

327 In conclusion, following a PRM challenge at 18.5 weeks, the serum levels of SAA were
328 significantly increased and this was subsequently found to be positively correlated with the
329 level of PRM infestation. Our proteomic investigation revealed a marked change in the levels
330 of two major proteins viz. Apolipoprotein B and Vitellogenin-2 in the sera of hens between
331 12 and 17 weeks of age. The serum proteome however did not appear to be significantly
332 altered in the weeks following the PRM challenge. It may be that the levels of mite
333 infestation were too low to stimulate a major APR or that the chronicity of the disease did not
334 show a clear proteomic change as the hens quickly acclimatized to the stress of the infestation.
335 Further work is needed to establish if serum proteins differ in high and low producing hens
336 and to determine if PRM infestation alters the serum protein profiles in older birds.

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345 throughout this project.

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Appendix B: - Reagents and buffers

Section 2.1

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.

Section 2.2

SDS-PAGE Running Buffer
144 g Glycine
30.3 g Tris
10 g SDS 282 Appendices
Add the glycine to 700ml of distilled water, once dissolved add Tris and SDS and finally made up the volume of 1L with dH2O.

Section 2.3

TTBS (10X)
60.2 g Tris-base
87.6 g Sodium Chloride (NaCl)
10 ml Tween 20
700 ml dh2O.
Adjust pH to 7.4 with 1M hydrochloric acid (HCl) and make the volume up to 1L.

Section 2.4

Transfer buffer (1X)
6.06 g Tris
28.8 g Glycine
1600 ml dH2O
400 ml Methanol
Mix well and adjust pH to 8.0 with 1M sodium hydroxide (NaOH) 284 Appendices

Section 2.5

Coating buffer
0.05 M Carbonate-Bicarbonate
Adjust pH to 9.6 with 1M sodium hydroxide (NaOH)

Section 2.6
TBS (Tris Buffered Saline) (x10)
Tris 22g
NaCl 72g
Dissolve in 1L dH2O and adjust to pH 7.6 with concentrated HCl

Section 2.7
ELISA wash and blocking solution
50 mM Tris
0.14 M NaCl
0.05% Tween 20
Adjust pH to 8.0 with 1M sodium hydroxide (NaOH)

Sample and conjugate diluent
50 mM Tris
0.14 M NaCl
0.05% Tween 20
Adjust pH to 8.0 with 1M sodium hydroxide (NaOH)

ELISA Stop Solution
0.18 M H_2SO_4

Section 2.8
RID gel
Dissolve 0.17g agarose in 17ml TBS
While in water bath (56˚C) add antibody, mix and pour immediately

Appendix C: - Tables

section 3.1

Table Proteomic analysis of bands1-11 in sera sampled at 17weeks of age.
Proteomic analysis of the proteins contained in bands 1-11 excised from a 1D-SDS-PAGE gel of pooled sera at 17 weeks of age Figure 4.8. For each band, the top three protein hits based on the MOlecular Weight SEarch (MOWSE) scores are presented. Characters in italics present proteins predicted from known genomic sequences. The full data set is presented as supplementary data.

Band	Protein [species]	Accession number	Mass	MOWSE Score	Number of peptides matched	Peptide Sequences	emPAI	pI	Sequence Coverage (%)
1	**Apolipoprotein B** precursor [Gallus gallus]	gi|113206052	524519	3838	362(156)	195(105)	0.83	8.51	40%
	Vitellogenin [Gallus gallus]	gi|212881	206862	345	40(13)	32(11)	0.18	9.22	17%
	Apolipoprotein B	gi|114013	50873	230	17(8)	14(6)	0.43	8.22	32%
2	**Vitellogenin-2** precursor [Gallus gallus]	gi|71896765	206732	3299	262(115)	94(57)	1.53	9.23	51%
	Vitellogenin-2-like, partial [Gallus gallus]	gi|971443652	34967	1073	58(30)	18(13)	2.32	8.95	66%
	Vitellogenin-1 precursor [Gallus gallus]	gi|52138705	212608	791	51(30)	39(27)	0.47	9.16	23%
3	***Alpha-2-macroglobulin** [Gallus gallus]*	gi|971373602	131990	952	85(39)	40(24)	0.78	6.19	35%
	Alpha-2-macroglobulin-like [Gallus gallus]	gi|971373692	166154	884	74(39)	29(19)	0.5	5.56	20%
	Vitellogenin [Gallus gallus]	gi|212881	206862	823	82(31)	50(24)	0.42	9.22	29%
4	**Vitellogenin-2** precursor [Gallus gallus]	gi|71896765	206732	785	85(32)	51(22)	0.42	9.23	28%
	Complement C3 precursor [Gallus gallus]	gi|45382303	185510	500	53(27)	28(17)	0.32	6.69	14%
	Vitellogenin-1 precursor [Gallus gallus]	gi|52138705	212608	182	25(7)	21(7)	0.11	9.16	12%
5	***Alpha-2-macroglobulin-like protein 1 [Gallus gallus]***	gi|971434955	164547	743	93(29)	51(24)	0.59	5.98	39%
	Vitellogenin [Gallus gallus]	gi|212881	206862	551	65(21)	36(14)	0.25	9.22	20%
	Vitellogenin, partial [Gallus gallus]	gi|50582493	163656	483	50(19)	28(12)	0.27	9.51	20%
6	**Chain A Ovotransferrin**	gi|83754919	77518	1338	136(46)	46(25)	1.88	6.7	69%
	Ovotransferrin Precursor	gi|1351295	79551	1338	135(46)	45(25)	1.8	6.85	63%
	Ovotransferrin BC type [Gallus gallus]	gi|71274079	79588	1302	127(46)	44(24)	1.7	7.08	66%
7	**Serum Albumin** precursor [Gallus gallus]	gi|766944282	71841	3889	278(129)	54(36)	6.56	5.51	87%
	Unnamed protein product, partial [Gallus gallus]	gi|63524	54462	422	36(15)	14(8)	0.56	6.84	31%
	Complement C3 precursor [Gallus gallus]	gi|45382303	185510	349	31(13)	18(10)	0.2	6.69	13%
8	**Serum albumin Precursor**	gi|113575	71868	593	72(16)	29(8)	0.46	5.51	48%
	Unnamed protein product, partial [Gallus gallus]	gi|63524	54462	273	24(8)	10(5)	0.32	6.84	20%
	Complement C3 precursor [Gallus gallus]	gi|45382303	185510	182	16(8)	9(6)	0.1	6.69	5%

9	***Keratin, type II*** *cytoskeletal 8 [Gallus gallus]*	gi|971446176	53214	292	17(8)	6(3)	0.19	5.36	11%
	Keratin 6A [Gallus gallus]	gi|762207165	57371	258	15(9)	5(3)	0.17	8.48	8%
	Serum albumin Precursor	gi|113575	71868	243	27(9)	13(7)	0.34	5.51	26%
10	**Serum albumin** Precursor	gi|113575	71868	357	44(15)	23(11)	0.66	5.51	40%
	keratin, type II cytoskeletal 8 [Gallus gallus]	gi|971446176	53214	111	8(3)	2(1)	0.06	5.36	5%
11	**Apolipoprotein AI** [Gallus gallus]	gi|227016	28790	885	90(34)	27(16)	4.83	5.45	89%
	Apolipoprotein A-I [Gallus gallus]	gi|211159	30673	885	89(34)	26(16)	4.24	5.58	78%
	Immunoglobulin lambda chain [Gallus gallus]	gi|1536804	10883	192	8(4)	4(2)	0.69	4.72	38%

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