Chapter 1: General Introduction

The Australian chicken meat industry is an important component of Australia's agricultural sector with in excess of 800,000 tonnes of chicken meat being produced annually and gross annual value of production exceeding $1.9 billion (ACMF, 2013). Chicken is now the most consumed meat in Australia which is remarkable given that leading into the 1970's Australia's chicken meat consumption was well below that of beef and lamb and slightly below that of pork. The change in consumption patterns of chicken meat in Australia are attributed to an increased range of marketed chicken products, improved quality and consistency, targeted marketing techniques and price competitiveness (ACMF, 2013). Key to the current price competitiveness of chicken meat is the efficiency feats achieved by the industry with marked improvements in both growth rates and feed conversion efficiencies. In 1975 the mean age (days) required to reach 2 kg live weight was 64.1 days compared to 35.0 days in 2010 (ACMF, 2013). During this period the feed conversion ratio (kg feed/kg liveweight) has been reduced from 2.33 to 1.70 (ACMF, 2013).

In 2010, an acute paralysis syndrome (APS) was observed in Australian broiler chickens characterised by flaccid paralysis of the neck, sternal recumbency and higher than normal flock mortality rates. Initial diagnostic testing ruled out virulent Newcastle disease (ND) and avian influenza (AI) as causes of the APS, and results were inconsistent with the APS being a form of botulism. From the initial observation in 2010, numerous cases were observed on different farms; however, all were located within one region. Descriptions of cases by farm personnel and veterinarians were that cases of the APS seemed to be more prevalent at warmer times of the year and in faster growing flocks, clinical signs were not seen before 26 days of age and that more males than females tended to be affected with the APS. Veterinarians reported that gross lesions were not observed in any organ system of affected chickens.

Further diagnostic investigations conducted by the State Veterinary Diagnostic Laboratory (SVDL) revealed histopathological lesions in the brain of a proportion of affected chickens indicative of vasculocentric encephalitis of the cerebrum and Wallerian degeneration throughout the spinal cord. Shed dust from a selection of farms was analysed for the presence and amount serotype-1 Marek's disease virus (MDV1) by quantitative polymerase chain reaction (qPCR) at the University of New England (UNE) (Armidale, NSW) in which high viral copy numbers (VCN) of MDV1 were detected. Furthermore, MDV1 was detected in spleen and brain tissues from a proportion of affected chickens.

The combination of the histopathological and qPCR findings directed veterinarians to make a tentative diagnosis of Marek's disease (MD) and specifically the acute transient paralysis (TP) form. The clinical appearance and histopathological findings specific to the brain were believed to most closely resemble TP as described by Kenzy *et al.* (1973), Swayne *et al.* (1989a), Witter *et al.* (1999) and Zander (1959). Following the tentative diagnosis of MD, vaccination of broilers with Herpesvirus of Turkeys (HVT) in the region commenced in 2012 with the vaccine being delivered *in-ovo* at day 18 of incubation. Cases of the APS continued to be observed despite HVT vaccination and a subsequent sharp decline in MDV1 levels in shed dust. MDV1 was then considered less likely to be the causative agent of the APS given the continued presence of the APS despite the sharp reduction in MDV1 load in shed dust subsequent to HVT vaccination. The investigations reported in this thesis commenced at this point in the history of the APS.

The primary purpose of the research undertaken and reported in this thesis was to determine aetiology of the APS, risk factors for it and management strategies for controlling it. Initially several broad hypotheses were made in relation to the APS, firstly that the APS had an infectious aetiology and that was unlikely to be MDV1 and secondly, that maternal antibody was important for early protection against that APS which explained the timing of the APS in the field. The study was multi-facetted in its approach with the major components being clinical and differential diagnosis assessment, farm-level epidemiological investigations, experimental reproduction of the syndrome and further diagnostic testing. Initially, the clinical assessment of affected chickens and the collation of diagnostic data allowed the APS to be appropriately described and for a differential diagnosis list to be established, both being essential components of any veterinary disease investigation. The aim of epidemiological investigations was to identify factors or combinations of factors which were associated with the presence of the APS in broiler flocks in order to assist with developing an understanding of likely causation, to direct further investigations and to identify mitigation strategies which could implemented on-farm in an attempt to reduce incidences of the APS.

Experimental investigations were initially designed to test whether the APS was infectious prior to attempting identification and isolation of any likely associated agent/s, in line with Evan's unified concept for causality (Evans 1976). Thus, the initial attempt at experimental reproduction of the APS tested whether the APS could be experimentally reproduced in broiler and specific pathogen free chickens using a variety of harvested challenge materials (spleen, blood, brain and litter) at two ages at challenge (day of hatch and 21 days later). Subsequent attempts at experimental reproduction tested the infectivity of different batches of harvested challenge material, the protective effect of exposure to contaminated litter at hatch, the infectivity of

challenge materials passaged through chickens in previous experiments, the effect of challenge dose and whether immunosuppression increased the susceptibility of chickens to the APS.

Finally, whole-genome next generation sequencing (NGS) was utilised in order to test for the presence of bacteria and/or viruses in affected chicken tissue not present in control chicken tissue. This technique whilst being relatively new is becoming more widely used in order to identify new infectious agents associated with veterinary disease.

The results presented in this thesis should provide industry with strategies which can be implemented on-farm to reduce the incidence of the APS whilst providing a basis for future work into experimental models for reproduction of the APS which are required for confirming the effectiveness of control strategies and the further association of infectious agents and risk factors.

Chapter 2: Review of the literature

2.1 Introduction

The purpose of this thesis is to establish the causation of an acute paralysis syndrome (APS) in broiler chickens as outlined in the General Introduction (Chapter 1). The review of literature herein focusses on the epidemiological and diagnostic features relevant to a thorough investigation of a veterinary disease of unknown aetiology. Section 2.2 explores historical and elementary aspects of veterinary disease epidemiology and causation. Section 2.3 explores approaches to establishing veterinary disease diagnosis, the procedure followed for a complete neurological examination of avian species and potential causes of neurological disease in poultry.

2.2 Veterinary epidemiology

2.2.1 Introduction to veterinary epidemiology

Veterinary epidemiology is concerned with disease in animal populations and specifically the frequency, distribution and determinants of the disease. The evolution of veterinary epidemiology and medicine has been documented since the establishment of the first veterinary school in France in 1762 (Thrusfield, 2005). Key to epidemiology is the value gained from the thorough investigation of a population of animals, rather than an investigation focussed on a small pool of diseased animals.

There are five primary objectives of epidemiology as set out by Thrusfield (2005):

1. Determination of the origin of a disease for which the cause is known;
2. Investigation and control of a disease for which the cause is either unknown or poorly understood;
3. Acquisition of information on the ecology and natural history of a disease;
4. Planning, monitoring and assessment of disease control programs; and
5. Assessment of the economic effects of a disease and analysis of the costs and economic benefits of alternative control programmes.

The epidemiological investigations into the APS are in their infancy with its cause being unknown and significant control programs yet to be implemented. Considering this, the 2nd and 3rd objectives of epidemiology are most relevant to subsequent investigations into the APS reported in this thesis.

Relevant to the 2nd objective of epidemiology, investigation and control of a disease for which the cause is either unknown or poorly understood; there are numerous examples of diseases against which control measures have been effectively implemented prior to the causation of the disease being established, including:

- The eradication of contagious bovine pleuropneumonia from the USA, from the observation of the infectious nature of the disease but prior to the isolation of *Mycoplasma mycoides* as the causative agent (Schwabe, 1984; Thrusfield, 2005).
- The legislation implemented throughout the world preventing the feeding of ruminant-derived protein to livestock was based on epidemiological analyses that linked the development of bovine spongiform encephalopathy (BSE) to the consumption of feedstuffs containing meat and bone meal contaminated with a scrapie-like agent (Thrusfield, 2005; Wilesmith *et al.*, 1988).
- Epidemiological studies linked the occurrence of squamous cell carcinomas of the eyes of Hereford cattle to the lack of pigmented skin associated with the eyelids (Anderson *et al.*, 1957; Thrusfield, 2005).

Conversely epidemiological analyses can assist in determining the cause of a disease, from which point control measures can be implemented. For example, Willeberg (1981) found that low levels of water consumption were a causative factor in feline urolithiasis. From this understanding control measures could be put into place, such as dietary modification to increase the water content of feeds offered.

The 3rd objective of epidemiology relates to gaining greater understanding of a disease by considering the interaction between factors relating to the host, pathogen and the environment. Host-pathogen-environment (HPE) considerations will be discussed further in this chapter (section 2.2.4).

There are four recognised approaches to epidemiological investigations, including:

- Descriptive epidemiology,
- Analytical epidemiology,
- Experimental epidemiology, and
- Theoretical epidemiology (Thrusfield, 2005).

Descriptive epidemiology often forms the first part of an epidemiological investigation. It can be subjective; however, observations made can form hypotheses which can be subsequently tested experimentally. Analytic epidemiology is less subjective than the descriptive approach, as the analysis of observations via suitable diagnostic and statistical methods are required. The experimental approach allows for the observation and analysis of data from a population, in which the factors associated with certain groups can be controlled and altered. The theoretical approach utilises mathematical models in order to simulate naturally occurring disease patterns.

2.2.2 Measuring disease frequency in animal populations

The effect of a disease in an animal population is typically spoken of in terms of mortality or morbidity. Mortality is the proportion of animals dying as a result of the disease whereas morbidity is the proportion of animals experiencing the disease. Prevalence and incidence are two commonly used measures of morbidity (disease frequency) in epidemiological studies. Prevalence is the proportion of animals in the population experiencing the disease at a set point in time and informs of the number of existing cases. Incidence is the proportion of a population initially free of a disease but which experience the disease over a specified time period and thus informs of the rate of new case development. The mortality rate is similar to incidence measures whereby death replaces a case in the calculations (Thrusfield, 2005).

Prevalence and incidence data are useful in three ways (described below) with the third point being most relevant to populations of animals:

1. To assign a probability that an animal has a disease;
2. To predict the clinical course of the disease for a patient; and
3. To make comparisons or measures of association between groups of animals with and without exposure to a risk or causative factor (Flanders *et al.,* 2016; Toribio, 2008b).

For incidence, the measure of association is relative risk, which indicates the chance of a disease occurring in an exposed group relative to the chance of a disease occurring in an unexposed group (Toribio, 2008b). Relative risk (RR) is calculated from the equation:

RR = Incidence in exposed / Incidence in unexposed.

For prevalence, the measure of association is the odds ratio (OR), which measures the odds of disease being present when a risk factor is present compared to the odds of disease being present when the risk factor is absent (Flanders *et al.,* 2016; Toribio, 2008b). The odds ratio OR is calculated from the equation:

OR = (# cases in exposed × # non-cases in unexposed) / (# non-cases in exposed × # of cases in unexposed).

2.2.2.1 Epidemiological studies

All epidemiological investigations commence with the collection of suitable data. There are a number of different approaches to epidemiological investigations available and the approach adopted is dependent on the type of data available and the level of understanding that already exists on the disease under investigation.

Surveys

In the context of veterinary epidemiology, a survey involves an examination of a group of animals (aggregate), for example, for the presence of a disease or for production parameters. A cross-sectional survey examines events in the aggregate occurring at one point in time, whereas a longitudinal survey examines events in the aggregate over a period of time. Screening is a particular type of survey (Thrusfield, 2005) and is characterised by the rapid identification of undiagnosed cases of disease using tests (e.g. serology) or examinations with the aim being to separate healthy individuals that most likely have the disease, from those that probably do not. Screening is an important component of the response to emergency disease outbreaks.

Monitoring

Monitoring involves making routine and standard observations on factors of interest in an animal population, for example, milk yield recording in the dairy industry.

Surveillance

Surveillance is a more intensive version of monitoring, whereby data is collated from monitoring programs and interpreted accordingly to monitor the health of a population over time. In surveillance programs the identities of diseased animals are typically recorded. An example of surveillance is the Sheep Market Assurance Program used in Australia for the surveillance of Ovine Johne's Disease in sheep flocks.

Studies

In epidemiology, studies involve the comparison of groups of animals within a population and they utilise a greater degree of analysis than would be performed in a survey. The four main types of epidemiological study include experimental, cross-sectional, case-control and cohort.

In experimental studies, the investigator randomly assigns factors (e.g. treatment) to animals or groups of animals in the study population. Experimental studies are different to the three other types of studies (cross-sectional, case-control and cohort) which are broadly classified as observational studies. The main difference between experimental and observational studies is that

in observational studies the investigator cannot randomly assign animals to groups. In an experimental study the investigator has greater control over the factors.

A cross-sectional study investigates the prevalence or number of cases of the disease in a population at a given point in time or over a given time period (Mann, 2003) in relation to hypothesised causative factors, so that associations can be drawn. Information is collected on a single occasion. As cross-sectional studies are relatively cheap and quick to perform, they can be repeated to assess changes over time. The main disadvantage in cross-sectional studies is that because disease and possible causative factors are measured simultaneously, it may be unclear as to which came first (Toribio, 2008b). Relative risk can be derived in relation to potential causative factors from cross-sectional studies, which defines the chance of disease occurring in the exposed group relative to the unexposed group.

A case-control study is designed to assess whether a risk factor (causative factor) is more common in animals with a given disease than in animals without. In contrast to cross-sectional and cohort studies, case-control studies are retrospective. They determine the relative importance of a factor in relation to the presence or absence of the disease under investigation. Case-control studies allow an OR to be derived (Mann, 2003) for each causative factor being investigated, which describes the relative odds of disease occurring in the exposed population, compared to that in the unexposed population.

A cohort study determines the incidence of a disease in a population, which is the number of new cases in a population over a given time. Representative animals (or farms) exposed to a suspected risk factor are selected along with a comparative group that is not exposed. At the end of the observation period the incidence of the disease is compared between the two groups and the relative risk of disease among individuals of the exposed group compared to the unexposed group can be reported. Cohort studies provide the strongest support for a causal association because they demonstrate a temporal association between exposure and disease (Toribio, 2008b).

2.2.2.2 Types of association

Association refers to the level of dependence or independence between two variables. There are two main types of association, non-statistical and statistical association (Thrusfield, 2005). A non-statistical association between a disease and a hypothesised causal factor is one of chance, for example, isolation of a contaminant bacteria from the eye of cats affected with conjunctivitis when such flora is subsequently found in the eyes of healthy cats. Variables are considered statistically associated when they occur in combination more frequently than would be expected by chance (Hernan, 2004) and thus can be indicative a causal relationship.

Interpretation of statistical association can be complicated by confounding, which is the effect of an extraneous variable that can wholly or partly account for an apparent association between variables (Fitzmaurice, 2003). Confounding variables are distributed non-randomly. An example of a confounding factor is described by Mackintosh *et al.* (1980), who found a statistical association between the wearing of milking aprons and large dairy herd sizes and the contracting of leptospirosis by dairy workers. However, they also found that dairy workers in larger herds were more likely to wear aprons than workers in small herds. The statistical association between wearing aprons and contracting leptospirosis was produced by the confounding effect of large herd size.

If cause of disease is regarded as a *necessary cause* if it must be present in order for disease expression to occur. Given that most diseases are multifactorial in aetiology, a cause is regarded as a *sufficient cause* if it inevitably results in disease and in most cases a *sufficient cause* has numerous component causes (Thrusfield, 2005).

An example of a multifactorial disease is that of actinobacillosis or 'wooden tongue' in cattle. The bacterium *Actinobacillus ligneresi* is the *necessary cause* of the disease but not the *sufficient cause*, as other factors that damage the mucosa of the tongue are required for disease induction, such as sharp/dry vegetation in periods of drought. So for 'wooden tongue' the *sufficient cau*se is the combination of *A. ligneresi* and the consumption of feedstuffs which damage the buccal mucosa.

2.2.3 Disease distribution

Descriptions of a disease typically include time (temporal) and geographic (spatial) distributions.

2.2.3.1 Temporal distribution of disease

Patterns of temporal distribution of a disease are often described as endemic, epidemic, pandemic, sporadic or an outbreak (Table 2.1). A single occurrence of a disease is described as an outbreak, whereas a disease which is described as endemic is considered to be at a stable state in a population and the level of disease is typically predictable, for example, cutaneous myiasis in Merino sheep. An epidemic can occur if a disease is introduced into a naïve population (e.g. foot-and-mouth disease) or there is a change in host and/or agent factors affecting the population that were previously not present (e.g. increased consumption of dry/sharp vegetation in drought conditions leading to an epidemic of actinobacillosis in beef cattle). Epidemics can also progress to endemic or sporadic distributions as the host adapts to the new disease or there are management interventions.

Table 2.1 Temporal descriptions of disease (Thrusfield, 2005)

Temporal distribution	**Description**
Endemic	The constant presence of a disease in a population, but may have a seasonal pattern
Epidemic	The occurrence of a disease (infectious or non-infectious) to a level in excess of endemic levels
Pandemic	A widespread epidemic, affecting a large proportion of the population
Sporadic	The irregular occurrence of a disease in a population
Outbreak	A single occurrence of a disease affecting multiple animals

There are three typical trends in the temporal distribution of disease, short-term, cyclical and long-term (Thrusfield, 2005). Short-term trends are typical of epidemics and are characterised in sequential order by a rise, peak and fall in disease incidence over a period of time. Cyclical trends are consistent with an endemic disease with regular and predictable cycling of disease incidence over time. Long-term trends reflect the interaction between the host and the agent and variations in disease incidences reflect the balance achieved between the host and the agent, for example incidence remains relatively constant over-time when equilibrium is achieved between the host and the agent, disease incidence rises over time when conditions favour the agent and disease incidence falls over time when conditions favour the host.

2.2.3.2 Spatial distribution of disease

Mapping is a commonly employed method for displaying the spatial distribution of a disease and hypothesised causal factors. McCrea and Head (1978) discovered a link between tumours in sheep (predominantly associated with the jaw) and bracken fern by drawing maps and observing clusters of cases in areas known to contain the plant. Mapping can additionally assist in investigating the direction of transmission of infectious diseases which may allude to modes of transmission (e.g. wind).

In epidemics, cases are observed to cluster in a defined area (Thrusfield, 2005) and epidemics associated with an infectious disease show a contagious spatial pattern which is obvious on mapping. This pattern contrasts with sporadic outbreaks in which the spatial pattern is seen to be random on mapping. As mentioned earlier, the observation of clusters of cases of a disease on mapping can be suggestive of causative factors of the disease.

2.2.4 Determinants of disease

In the late 19th century Robert Koch, the renowned German bacteriologist, formulated his postulates of causation of infectious disease, which stipulated that for an infectious agent to be identified as a cause of a disease:

- It must be present in all cases of the disease;
- It must not occur in another disease as a fortuitous and non-pathogenic parasite; and
- It must be isolated in pure culture from an animal, be repeatedly passaged and subsequently be able to induce the same disease in other animals (Thrusfield, 2005).

It is understood that Koch considered the teachings of his then professor, Jakob Henle in formulating his postulates (Evans, 1976). The combined work of Henle-Koch was the first to provide a framework for guided investigation into infectious diseases of animals. Diseases of great importance in animals at the time included anthrax, tuberculosis, erysipelas and tetanus (Evans, 1976) as these pathogens were seemingly capable of causing disease irrespective of environmental conditions. As such, Koch's postulates were valid in these cases. However, one of the major flaws in this thinking is that it failed to account for multifactorial components of disease. Disease causation is more often multifactorial than not. In 1937, the American virologist Thomas Rivers noted that Koch's postulates posed limitations in investigating disease causation with respect to viruses as aetiological agents. Rivers proposed conditions for establishing a relationship between a virus and a particular disease which included:

- A specific virus must be found associated with a disease with regularity;
- The virus must be shown to occur in a sick individual as the cause of the disease, for example, in cases of sickness the virus must be shown to occur in specific lesions at specific times; and
- When establishing proof of causation, animals should become sick when inoculated with tissue from lesions containing the virus, when such tissue is shown to be free of other infective agents (Evans, 1976; Rivers, 1937).

Rivers recognised that some animals will carry certain viruses asymptomatically, and that such may pose interpretation difficulties when attempting to prove a causal relationship by reproducing disease. In addition, Rivers went on to discuss the importance of the presence of antibodies to the agent of interest in blood serum and the timing of their presence in relation to inoculation (Rivers, 1937). Rivers suggested that if a virus is causing a disease, serum antibodies to that virus should be absent at the clinical stage of disease and be present more in the recovery stage (Rivers, 1937).

The concept of the need for epidemiological data to highlight statistical association in investigating causation was discussed by Robert Huebner in 1957 (Huebner, 1957). This was at a time when new viruses were being identified and isolated frequently due to technological improvements and such viruses were commonplace in both sick and clinically normal humans (Evans, 1976). Huebner suggested that the following conditions must be met in order to identify a particular virus as the cause of a human disease:

- The virus must be well established by passage in the laboratory;
- The virus must be repeatedly isolated from human specimens and shown not to be a contaminant of the cells/media used to grow it;
- An increase in neutralising or other antibodies should regularly result from active infection;
- A new virus should be fully characterised and compared with other similar agents;
- The virus must be constantly associated with any well-defined clinical syndrome;
- Humans being inoculated with the agent in double blind studies should reproduce the clinical syndrome;
- Epidemiological causation should be shown through longitudinal and cross-sectional studies; and
- Vaccination should provide a protective effect (Huebner, 1957).

With advances in technology through the 1960's and 1970's, particularly with the introduction of the electron microscope and serological techniques, infective agents could be identified in humans and animals without investigators being able to isolate and propagate them. In this scenario it was not possible to apply the prior postulates of Koch, Rivers and Huebner to determine causation. It was at this time that investigators began to link serological and immunological characteristics (as first discussed to some degree by Rivers) associated with infection to causation. One such example was when Werner Henle (the grandson of Jakob Henle) in 1968 claimed Epstein-Barr virus was the cause of infectious mononucleosis, based on immunological findings (Evans, 1976).

These immunologic and serologic criteria for determining causation cannot be applied in certain chronic viral infectious of the central nervous system (CNS), as a very lengthy incubation period is often a feature of such infections. Considering such, Johnson and Gibbs (1974) proposed criteria for relating slow viral infections to chronic neurological disease. Establishing causation for virus-induced cancer poses similar challenges. The often lengthy incubation periods, relatively low incidence of most cancers, phenomenon of reactivation of viruses leading to cancer, ubiquitous nature of oncoviruses and great difficulty in experimental replication can make the

establishment of causation difficult. In these cases, the protective effect afforded by vaccination has been considered good enough evidence of causation, for example, the prevention of tumours in chickens after the administration of Marek's disease virus (MDV) vaccine early in life, provides strong evidence favouring causation of tumour by the virus (Evans, 1976).

There are conditions in a whole range of situations in which investigating and attempting to prove causation are difficult. These include:

- The same pathologic or clinical disease can be produced by different agents;
- Aetiological agents can vary over different geographic areas, host age groups and host susceptibility factors;
- Some diseases require the presence of multiple infective agents, or factors in conjunction;
- One agent can produce different clinical and pathological syndromes in different settings;
- There is often a gradient of changes and symptoms in any disease; and
- Variations in host response to infection can have an effect on disease progression (Evans, 1976).

Given the conditions outlined in the above points and based on the history since the early work of Henle-Koch, Alfred Evans was prompted to devise his unified concept in setting criteria for causation, which includes:

1. *Prevalence* of the disease should be significantly higher in those exposed to the putative cause than in controls not exposed;
2. *Exposure* to the putative cause should be present more commonly in those with the disease than in controls without the disease when all risk factors are held constant;
3. *Incidence* of the disease should be significantly higher in those exposed to the putative cause than in those not so exposed as shown in prospective studies;
4. *Temporally,* the disease should follow exposure to the putative agent with a distribution of incubation periods on a bell shaped curve;
5. *A spectrum* of host responses should follow exposure to the putative agent along a logical biologic gradient from mild to severe;
6. *A measurable host response* following exposure to the putative cause should regularly appear in those lacking this before exposure or should increase in magnitude if present before exposure, this pattern should not occur in animals or persons not so exposed;
7. *Experimental reproduction* of the disease should occur in higher incidence in animals or man appropriately exposed to the putative cause than in those not so exposed, this

exposure may be deliberate in volunteers, experimentally induced in the laboratory, or demonstrated in a controlled regulation of natural exposure;

8. *Elimination or modification* of the putative cause or of the vector carrying it should decrease the incidence of the disease;
9. *Prevention or modification* of the host's response on exposure to the putative cause should decrease or eliminate the disease;
10. The whole thing should make biologic and epidemiologic sense (Evans, 1976).

When considering the need to demonstrate epidemiological association in defining causality, Lower (1983) made the important point that statistically significant association when considered alone does not prove causality, rather logical reduction of proof needs to be considered, whereby disease induction and the subsequent chain of events need to be explained to provide a better understanding of the deemed causality. This is consistent with Evans' 10th concept of causation, requiring consideration of the interplay between the host, the pathogen and the environment when attempting to understand a disease and establish causation. Variations in agent pathogenicity, ability of the host to respond to the pathogen and in environmental conditions must all be considered and the established web of causation must make biological and epidemiological sense.

For most diseases the presence of a causative factor (e.g. infectious agent) is necessary but not usually sufficient to cause disease. Rather, in most diseases, expression is a result of the complex interplay between a range of factors specific to the host, the pathogen and the environment, which are often referred to as the 'HPE triad'. Table 2.2 summarises the common HPE determinants for disease expression.

Table 2.2 HPE determinants for disease expression (Thrusfield, 2005; Walkden-Brown, 2014)

Host determinants	**Pathogen determinants**	**Environmental determinants**
Genotype	Virulence and pathogenicity	Location
Age	Antigenic variation	Climate
Sex	Reproductive potential	Husbandry
Species/breed	Dispersal efficiency	Stress
Size/conformation	Survival efficiency	Presence of vectors/reservoirs

2.2.4.1 Maintenance and transmission of infectious disease

Infectious disease requires the invasion of a susceptible host by the pathogenic organism. Continued survival of the infectious agent requires continual transmission, infection and replication in a population of susceptible hosts. This process is referred to as the life history (life-cycle) of the infectious agent (Thrusfield, 2005). Detailed understanding of the life history of an infectious agent is essential, in most cases, for effectively controlling disease associated with that agent.

Transmission of agents between hosts can occur either horizontally or vertically. Horizontal transmission is characterised by transmission from any segment of a population to another (Thrusfield, 2005), for example, chicken-chicken within a broiler shed. Horizontal transmission occurs by direct contact between primary hosts, with discharges from hosts being a common method of direct contact (e.g. respiratory droplets). Indirect horizontal transmission requires an intermediate vector to assist with transmission between primary hosts and this vector can be living or inanimate (e.g. farm equipment) (Thrusfield, 2005). An example of a living vector is a biting insect which acts as a vector in the transmission of arboviruses between hosts (e.g. Kunjin virus).

There are two types of vertical transmission, hereditary and congenital (Thrusfield, 2005). Diseases transmitted hereditarily are carried within the genome of either or both parents and transferred to the offspring (e.g. retroviruses) (Thrusfield, 2005). Congenitally transmitted diseases are transmitted to the next generation *in utero* or *in ovo* (Thrusfield, 2005).

2.3 Neurological disease in broiler chickens

This section will initially discuss the approach typically taken by veterinarians in formulating a list of possible diseases considered as potential diagnoses for a case under investigation, referred to as differential diagnosis list. In section 2.3.2 the neurological evaluation of avian species will be reviewed, as such is required for clinical evaluation of broiler chickens affected with the APS relevant to this thesis. Finally, section 2.3.3 will devise a broad differential diagnosis list, which is required for the investigation into the APS.

2.3.1 Veterinary disease diagnosis

Veterinarians consider a disease in an individual animal to be any variation in the normal functioning and/or structure of a body part, organ or body system, which manifests with a consistent set of symptoms (Myers & McGavin, 2007). Importantly, the aetiology and pathogenesis of a disease needs not be understood for the disease classification to be applied. Understanding of the aetiological and pathological features of a disease is gained from detailed epidemiological investigations.

In attempting to reach a diagnosis for a particular case, a veterinarian will often devise a differential diagnosis list, which essentially is a list of possible diseases that need to be individually ruled out in order for a definitive diagnosis to be reached. Reference is often made to different levels of a diagnosis, which basically describes how a particular diagnosis was reached. For example, a clinical pathological diagnosis relates to evidence of changes in haematology and biochemistry; a morphological diagnosis relates to evidence of the predominant lesions (macroscopic and/or microscopic), which is closely linked to a particular disease; and an aetiological diagnosis relates to evidence of the causative agent of the disease. In some cases a definitive diagnosis cannot be reached if, for example, diagnostic tests for the causative infectious agent are not available or affordable. In these situations, treatment and/or control strategies are often implemented in the absence of a definitive diagnosis; rather, they are based on a tentative or provisional diagnosis. Response to treatment is often used in these situations to confirm a diagnosis.

In reaching a diagnosis, investigation needs to go beyond the clinical examination of the host and consider pathogen and environmental factors. For example, in the investigation of an outbreak of calf scours, it would be important to look at housing, hygiene and dietary factors in addition to a clinical examination of affected animals, before making a diagnosis of the likely causative agent (or factor).

2.3.2 Neurological evaluation of avian species

2.3.2.1 Observation

The first aspect of the avian neurological examination is a general observation of the bird, taking note of mentation, posture, attitude and gait. Consciousness relies on effective functioning of the cerebrum and the brain stem (Fenner, 1995). Changes in mentation range from obtundation where the bird tends to sleep in an undisturbed state but can be aroused easily, stuporous where the bird requires noxious stimulation for arousal and coma, which is a state where voluntary responses cease, but neurological reflexes remain intact (Clippinger *et al.*, 1996). These three changes in mentation result from damage to the associated neurological pathways in the brain stem and cerebrum, as opposed to metabolic or systemic disorders which may induce signs of depression and lethargy in an affected bird due to its illness (Clippinger *et al.*, 1996).

Posture of the normal standing bird is characterised by an erect head, folded wings and wide based stance. The maintenance of this posture requires effective interaction between sensory receptors and motor activators in the peripheral nervous system (PNS) and integration into the CNS (Clippinger *et al.*, 1996). As a result of failure to maintain correct posture, recumbency will often occur, which can be caused by systemic illness (lethargy), pain or neurological dysfunction. Recumbency induced by neurological dysfunction often results from changes in muscle tone, from either increased rigidity or flaccidity. Muscle flaccidity may be observed as a result of a lower motor neuron (LMN) lesion and is indicative of a lesion in the nerve connecting the affected muscle to the CNS (Clippinger *et al.*, 1996); however, flaccidity may also be observed from generalised weakness, which is not anatomically indicative of lesion location. Increased muscle tone (rigidity) is an upper motor neuron (UMN) characteristic and is indicative of a lesion in the CNS cranial to the affected area (Clippinger *et al.*, 1996).

Attitude is considered a component of posture and describes the position of the eyes, head and limbs with respect to the body. Attitude is specific to the vestibular system, which has both central (cerebellum, brain stem and spinal cord) and peripheral (inner ear and vestibulocochlear nerve) components (Clippinger *et al.*, 1996). Abnormalities in attitude can result in nystagmus, head tilt, falling, rolling and circling (Clippinger *et al.*, 1996).

Normal movement and gait require both coordination and strength. Coordinated voluntary limb movements require effective functioning of the cerebral, cerebellar, vestibular and proprioceptive pathways (Clippinger *et al.*, 1996). The cerebrum controls precise movements (Fenner, 1995) and as such a cerebral lesion may induce hypermetric or hypometric gait changes. Balance abnormalities observed in bird movements are induced by lesions in the vestibular system. Weak

or absent limb movements are a result of dysfunction of neurological pathways in the cerebrum, brain stem, spinal cord and peripheral nerves (Clippinger *et al.,* 1996).

2.3.2.2 Cranial nerves

Following from observation of the affected bird as previously discussed, cranial nerve assessment is a necessary component of a thorough neurological examination. As each cranial nerve exits the brain at a distinct location, cranial nerve assessment can assist in localising lesions to a specific region of the brain, therefore all 12 cranial nerves should be assessed in sequence. Table 2.3 describes the function of each cranial nerve, signs of dysfunction and the tests which are used to assess functioning of each.

Evaluation of the eye allows for assessment of the optic, oculomotor, trochlear, abducens and trigeminal cranial nerves. Intact vision, thus the ability to avoid obstacles, requires integrity of the optic nerve and associated cerebral pathways (Clippinger *et al.,* 1996). The menace response is a learned response and requires intact optic (to observe danger) and trigeminal (to close the eyelid to protect the eye) nerves. The pupillary light response in birds is often not as useful as in mammals given that the former have striated muscle in the iris conferring some control over pupil size independent of light intensity (Platt, 2006). This response requires correct sensory function of the optic nerve and parasympathetic functions of the oculomotor nerve (Clippinger *et al.,* 1996). The glossopharyngeal, vagus, accessory and hypoglossal nerves have significant anastomoses between distal nerve fibres which make identification of specific nerve involvement difficult, for example, regurgitation could indicate a lesion in any or all of the last four cranial nerves (Clippinger *et al.*, 1996).

2.3.2.3 Postural reactions

The CNS and the PNS are required for the maintenance of posture, whereby the PNS provides inputs in order to adjust muscle tone to maintain balance and the CNS provides inputs to coordinate and smooth muscle movement (Clippinger *et al.,* 1996). The most common approach to testing postural reactions in a bird is to move a wing or leg to an abnormal position and observe whether the bird is able to correct such movement (Platt, 2006). Postural deficits are observed at the level of or caudal to the site of the lesion (Platt, 2006). Ascending sensory pathways in the spinal cord are located in the outermost areas and thus are susceptible to compression injury (De Lahunta, 1983). Consequently, proprioceptive deficits often present prior to or in the absence of motor function deficits as pathways relevant to the latter are situated deeper in the spinal cord.

Table 2.3 Cranial nerves and their function in avian species: neurological tests used to detect dysfunction and relevant clinical signs of dysfunction, adapted from Clippinger et al., (1996) and Platt, (2006)

Cranial nerve	**Function**	**Tests**	**Signs of dysfunction**
I – Olfactory	Smell	Odour	Impaired smell (change in appetite or feeding behaviour) – not routinely evaluated
II – Optic	Vision	Menace	Impaired sight
III – Oculomotor	Control of extrinsic ocular muscle and the upper eyelid muscle	Eyeball position Menace Pupillary light reflex	Ventrolateral deviation of eyeball Drooped eyelid Dilated pupil unresponsive to light
IV – Trochlear	Control of extrinsic ocular muscles	Eyeball position	Dorsolateral deviation of the eyeball
V – Trigeminal	Facial and beak sensation Beak movement	Skin touch Palpebral Pinch jaw	Facial hyperaesthesia Wide palpebral fissure Unable to close jaw
VI – Abducens	Control of the extrinsic ocular muscle and third eyelid	Eyeball position	Medial deviation of the eyeball Third eyelid immobility
VII – Facial	Control of the muscles of facial expression	None applicable	Asymmetry of face
VIII – Vestibulocochlear	Control of hearing, balance and coordination	Startle	Impaired hearing Nystagmus Head tilt
IX – Glossopharyngeal	Control of the pharynx, larynx, crop and syrinx	Gag reflex	Dysphagia Voice loss
X – Vagus	Control of the muscles of the larynx, pharynx, oesophagus and crop	Gag reflex	Regurgitation Voice change Crop immotility
XI – Accessory	Control of the superficial neck muscles	None applicable	Poor neck movement
XII - Hypoglossal	Control of the muscles of the tongue, trachea and syrinx	Tongue grab/inspection	Tongue deviation

2.3.2.4 Spinal reflexes

Intact spinal reflexes require correct functioning of a sensory nerve which transmits to the spinal cord and a motor nerve which innervates muscle. This reflex arc does not involve the brain or other sections of the spinal cord; however, the brain may modulate this reflex activity through its ascending and descending pathways (Clippinger *et al.,* 1996). If there is a lesion in the motor component of a spinal reflex it is described as LMN and results in hyporeflexia or areflexia, which can be from damage to one or both of the sensory and motor components (Clippinger *et al.,* 1996). Hyperreflexia results from a lesion in the proximal modulatory pathways (from brain to reflex arc) and such is defined as UMN (Clippinger *et al.,* 1996).

The number of spinal nerves in birds varies between species; however, a pair of spinal nerves is associated with each vertebral segment. Each of these spinal nerves is comprised of a sensory (dorsal) and motor (ventral) root. Spinal cord enlargements divide the spinal cord and its spinal nerves into distinct regions including the cervical region, brachial plexus, thoracic region and lumbosacral plexus. It is at these enlargements that the spinal nerves join to form peripheral nerves (Clippinger *et al.,* 1996). The brachial plexus innervates the thoracic wall and wings. The lumbosacral plexus innervates the caudal abdominal body wall, legs, vent and tail (Bolton, 1971; Clippinger *et al.,* 1996).

Evaluation of spinal reflexes in birds is typically difficult due to the inability to have the bird in a relaxed and laterally recumbent position with minimal restraint, as is considered ideal for mammals. The evaluation typically begins caudally and moves cranially. The spinal reflexes that can often be evaluated in birds are as follows:

- Vent-sphincter reflex: this evaluates the caudal segments of the spinal cord and requires pinching/pricking of the vent which should induce contraction of the vent and tail movement (Platt, 2006). Flaccidity of the vent indicates a LMN lesion whereas a hypertonic vent indicates an UMN lesion.
- Pedal flexor (withdrawal) reflex: this evaluates the lumbosacral plexus and requires pinching of a toe or skin of the foot to illicit withdrawal of the leg in a normal bird (Platt, 2006). Hyporeflexia or areflexia is indicative of a LMN lesion and specifically the ischiatic nerve whereas, hyperreflexia is indicative of an UMN lesion. Extension of the contralateral leg during this reflex is abnormal and termed a positive crossed extensor reflex and resultant from an UMN lesion (Clippinger *et al.,* 1996).

- Patellar reflex: this evaluates the lumbosacral plexus and requires the application of a tap stimulus to the straight patellar tendon or straight quadriceps tendons. The stimulus stretches the *femorotibialis* muscle which stimulates the femoral nerve inducing muscle contraction and extension of the stifle (Platt, 2006). Hyporeflexia or areflexia is indicative of a LMN lesion, whilst hyperreflexia is indicative of an UMN lesion.
- Wing withdrawal reflex: this evaluates the brachial plexus and requires pinching of the major digit at the point of primary flight feathers (Platt, 2006). Similar to the pedal flexor reflex, hyporeflexia or areflexia are indicative of a LMN lesion. Hyperreflexia is indicative of an UMN lesion and extension of the contralateral wing during this reflex is abnormal and termed a positive crossed extensor reflex (Clippinger *et al.*, 1996).

2.3.2.5 Nociception (deep pain perception)

Assessment of deep pain perception should be performed in birds that demonstrate spinal cord disease as evidenced by proprioceptive and/or spinal reflex deficits and abnormalities of gait (Platt, 2006). The nociceptive pathways travelling to the brain are located deep within the spinal cord (De Lahunta, 1983) and their presence or absence can be indicative of the severity of spinal cord injury. The presence of a deep pain response requires not only that these deep spinal pathways are intact but also the cerebral perception of pain (Clippinger *et al.*, 1996). In order to assess the deep pain response, stimulation of a foot should begin with a light touch and progress to a hard pinch (Clippinger *et al.*, 1996). Conscious perception of pain is important in distinguishing such from an intact spinal withdrawal reflex and thus there should be presence of vocalisation, looking around at the site of pain or attempt to escape, in order to demonstrate such perception (Clippinger *et al.*, 1996).

2.3.2.6 Localisation of a lesion

The neurological examination is designed so that a lesion can be first localised to a major division within the nervous system: brain, spinal cord or PNS. Lesions localised to the brain should then be localised to the cerebrum, diencephalon, mid-brain, pons and medulla, cerebellum or vestibular system. Similarly lesions identified as being in the spinal cord should be localised to a major subdivision – cervical region, brachial plexus, thoracic region or lumbosacral plexus. Table 2.4 describes the possible clinical signs that can be associated with lesions in each of the defined intra-cranial and spinal regions.

Table 2.4 Possible clinical signs associated with lesions of the major divisions of the avian brain and spinal cord, adapted from Clippinger et al. (1996) and Platt (2006)

Location	**Normal function**	**Possible clinical signs associated with lesion**
Cerebrum	Controls learned and sensory activities including vision, touch and pain	Altered mental state, seizures, behavioural change, head pressing, central blindness
Diencephalon	Controls autonomic visceral functions and endocrine regulation	Altered mental state, seizures, behavioural change, endocrine disturbances, abnormalities of temperature
Midbrain	Controls alertness and CN III-IV function	Altered mental state, opisthotonus, contralateral paresis, ipsilateral pupil dilation and/or ventrolateral strabismus, contralateral dorsomedial strabismus
Pons and medulla	Controls CN V-XII and cardiorespiratory centres	Altered mental state, ipsilateral paresis, irregular respiration, decreased beak strength, decreased palpebral reflex, decreased facial sensation, third eyelid protrusion, medial strabismus, decreased facial muscle tone, decreased lacrimation, deafness, decreased vestibular function, dysphagia, regurgitation, tongue deviation
Cerebellum	Controls coordination	Normal mental state, normal strength, opisthotonus, dysmetric ataxia, intention tremors of the head
Vestibular system (central and peripheral)	Controls balance	Head tilt, nystagmus, ataxia, rolling, circling
Cervical region	Associated with spinal nerves innervating the head and neck	Normal cranial nerve function, UMN wing, UMN leg, UMN vent, neck pain, loss of pain perception (rare)
Brachial plexus	Associated with spinal nerves innervating the thoracic wall and wings	Normal cranial nerve function, LMN wing, UMN leg, UMN vent,
Thoracic region	Associated with spinal nerves innervating abdominal viscera	Normal cranial nerve function, normal wing reflex, UMN leg, UMN vent, potential kyphosis, potential spinal ± abdominal pain, loss of pain perception in limbs (variable)
Lumbosacral plexus	Associated with spinal nerves innervating the caudal abdominal body wall, legs, vent and tail	Normal cranial nerve function, normal wing reflex, LMN leg, LMN vent, loss of sensation in legs and vent (variable)

2.4 Differential diagnosis for neurological conditions of broiler chickens

In this component of the review diseases which are known to induce neurological deficits in broiler chickens will be considered. For this purpose, a broiler chicken is defined as any chicken grown for the purpose of slaughter for human chicken meat consumption. The age range considered for such a definition is from hatch until approximately 56 days of age. A broad differential diagnosis list is presented in Table 2.5 which also includes diseases associated with lameness, the reason being that mild or early stages of the APS induce gait abnormalities, which appear similar to those evident in cases of lameness. Chapter 4 will then take this broad differential diagnosis list and narrow it down with the assistance of diagnostic investigations.

Table 2.5 Differential diagnosis list for the acute paralysis syndrome under investigation

Disease	Clinical features relevant to the CNS and gait	CNS pathology?	Brain & Spinal Cord histopathological features
Viral Diseases			
Marek's disease (Transient paralysis form)	Classical TP characterised by varying degrees of ataxia and flaccid paralysis of neck or limbs, with recovery within 1-2 days; acute TP results in death within 72 hours of the onset of paralytic signs; disease typically presents 5-7 weeks of age (Schat & Nair, 2008; Swayne *et al.*, 1989a).	Yes	Brain: vasculitis resulting in vasogenic brain oedema associated with clinical paralysis. Perivascular cuffing, lymphocytosis and gliosis also often present (Swayne *et al.*, 1989a; Swayne *et al.*, 1989c). Spinal Cord: no reported abnormalities in this form of MD.
Marek's disease (Classical fowl paralysis form)	Asymetric paresis progressing to spastic paralysis of one or more of the extremities.	Yes	Brain & Spinal Cord: mild perivascular cuffing accompanied by gliosis; however, often no detectable lesions (Gimeno *et al.*, 2001). These early inflammatory lesions may often be followed by lymphoproliferative lesions (Schat & Nair, 2008).
Avian encephalomyelitis	Ataxia and increased tendency to sit, progressing through to rapid but fine tremors of the head and neck and lateral recumbency, which is worsened by exercising of affected chickens; disease is typically present at 1-2 weeks of age (Calnek, 2008).	Yes	Brain & Spinal Cord: diffuse, non-purulent encephalomyelitis, with obvious mononuclear perivascular infiltration and disseminated microgliosis (Calnek, 2008).
Newcastle disease	Muscle tremors, torticollis, paralysis of the legs and wings and opisthotonus can be observed from strains of vND in the neurotropic velogenic strains, but rarely in the mesogenic strains (Alexander & Senne, 2008).	Yes	Brain & Spinal Cord: non-purulent encephalomyelitis, gliosis, perivascular infiltration of lymphocytes, and hypertrophy of endothelial cells; neuronal degeneration is an obvious feature; lesions typically in cerebellum, brain stem and spinal cord; and rarely in the cerebrum (Alexander & Senne, 2008).
Avian influenza (High Pathogenicity)	Muscle tremors of the head and neck, inability to stand, torticollis, opisthotonus, and other abnormal head, neck and leg positions (Swayne & Halvorson, 2008).	Yes	Brain: lymphocytic meningoencephalitis, gliosis, and neuronal necrosis and neuronophagia; oedema and haemorrhage are less commonly seen (Swayne & Halvorson, 2008). Spinal Cord: lesions not commonly reported.
Arbovirus infection (e.g. West Nile Virus)	Tremors and paralysis (Reagan *et al.*, 1952).	Yes	Brain: non-suppurative encephalitis reported in experimentally inoculated chickens (Senne *et al.*, 2000). Spinal Cord: no reported lesions.

Disease	Clinical features relevant to the CNS and gait	CNS pathology?	Brain & Spinal Cord histopathological features
Reovirus (viral arthritis)	Lameness, with mobility often prevented due to hock joint immobilisation (one or both), swelling of gastrocnemius, digital flexor and metatarsal extensor tendons bilaterally or unilaterally; abnomal neurological signs are not a feature; disease is typically present at 3-4 weeks of age (Jones, 2008).	No	n/a
Avian leukosis virus	Paralysis can result with renal tumours impinging on the sciatic nerve (Fadly & Nair, 2008).	Yes	Brain: cerebellar hypoplasia, disorganisation of purkinje cell layer of cerebellum, absence of granular cells of internal granular layer of cerebellum (Toyoda *et al.,* 2006). Spinal Cord: abnormal lesions not reported.
Bacterial Diseases			
Salmonellosis (e.g. *Salmonella enterica* spp. a*rizonae; Salmonella typhimurium)*	Increased tendency to sit, paralysis, torticollis, opisthotonus and convulsions (Shivaprasad, 2008).	Yes	Brain: meningitis, infiltration of heterophils, presence of fibrin and bacterial colonies (Shivaprasad, 2008). Spinal Cord: abnormal lesions not reported.
Escherichia coli osteomyelitis/synovitis	Unresponsiveness at advanced stages of septic disease, lameness which depending on whether such is due to bilateral or unilateral infection can result in a hopping gait or can render the affected bird non-ambulatory.	Yes	Brain & Spinal Cord: meningitis and encephalitis is possible in septic disease but rarely reported (Barnes *et al.,* 2008).
Fowl cholera (*Pasteurella multocida*)	Torticollis is rarely seen in chronic cases of fowl cholera (Glisson *et al.,* 2008).	Yes	Brain: evidence of meningitis. Spinal Cord: abnormal lesions not reported.
Mycoplasma synoviae	Evidence of lameness, and swelling of joints, most pronounced in the hocks and feet.	No	n/a
Botulism (*Clostridium botulinum*)	Increased tendency to sit and reluctance to move, progressing through to complete flaccid paralysis of the legs, wings, neck and eyelids, with signs tending to progress from caudal to cranial. Dyspnoea can often be seen. Botulism typically affects broilers from 2-8 weeks of age (Clark, 1987).	No	n/a

Disease	Clinical features relevant to the CNS and gait	CNS pathology?	Brain & Spinal Cord histopathological features
Arthritis, synovitis, and osteomyelitis caused by other bacteria (*Staphylococcus sp.* and others)	Reluctance to walk and increased tendency to sit, lameness unilaterally or bilaterally, droopiness of wings (Mutalib *et al.*, 1983).	No	n/a
Listeriosis (*Listeria monocytogenes*)	Encehalitic form characterised by depression, ataxia, torticollis and opisthotonos (Cooper, 1989).	Yes	Brain: micro-abscessation and perivascular cuffing associated, and occasional axonal degeneration and gliosis most prominent in the brain stem (Cooper, 1989). Spinal Cord: lesions not reported.
Epidemic infectious spinal necrosis (*Enterococcus cecorum*)	Progressive degrees of lameness through to apparent paralysis, typically commencing with increased tendency to sit on hocks, arching of the back, through to permanent lateral recumbency (Martin *et al.*, 2011).	No	n/a
Enterococcal encephalomalacia (*Enterococcus durans*)	A range of neurological signs have been reported including incoordination, paralysis, leg tremors, prostration, rolling, opisthotonus, torticollis and circling (Cardona *et al.*, 1993).	Yes	Brain: malacia of the medulla oblongata and cerebellum (Cardona *et al.*, 1993). Spinal Cord: no reported lesions.
Other Infectious Diseases			
Mycotic encephalitis (*Aspergillus sp.*)	Torticollis and ataxia (Veen, 1973).	Yes	Brain: granulomatous encephalitis of the cerebrum and cerebellum (Charlton *et al.*, 2008). Spinal Cord: no reported lesions.
Toxoplasmosis (*Toxoplasma gondii*)	Ataxia, trembling, opisthotonos, torticollis, and apparent blindness (Biancifiori *et al.*, 1986; Bickford & Saunders, 1966).	Yes	Brain: perivascular cuffing of plasma cells, lymphocytic infiltration of choroid villi, ependymal proliferation of lateral ventricle, thickening of leptomeninges, gliosis and the presence of cysts in the cerebrum, brain stem and cerebellum (Bickford Saunders, 1966). Spinal Cord: no reported lesions.
Avian malaria (*Plasmodium sp.*)	Ataxia, opisthotonos, paralysis, and convulsions (Macchi *et al.*, 2010).	Yes	Brain: diffuse oedema and vacuolation/spongiform appearance; congestion and inflammation associated with meninges; congestion of blood vessels diffusely through brain tissue; evidence of necrosis, inflammatory cell infiltration and gliosis throughout brain tissue (Macchi *et al.*, 2010). Spinal Cord: no reported lesions.

Disease	Clinical features relevant to the CNS and gait	CNS pathology?	Brain & Spinal Cord histopathological features
Nutritional Diseases			
Vitamin D Deficiency	Lameness due to rickets - reluctance to walk, increased tendency to squat on hocks, often swaying from side to side, softening of bone, with onset of signs from 2-3 weeks of age (Klasing, 2008).	No	n/a
Vitamin E Deficiency	Ataxia and paresis, abnormal head positions progressing through to complete prostration and death, from 15-30 days (Klasing, 2008).	Yes	Brain: evidence of neuronal degeneration and severe oedema in the cerebrum and cerebellum, strong evidence of ischaemic necrosis (Klasing, 2008). Spinal Cord: no reported lesions.
Thiamin (Vitamin B1) Deficiency	Initially chickens from 2 weeks of age display an unsteady and paretic gait, which progresses through to muscle paralysis of the toes, legs, wings and neck such that chicks often adopt a 'star-gazing' position (Klasing, 2008).	No	n/a
Riboflavin (Vitamin B2) Deficiency	From 2 weeks of age, chickens are weak, reluctant to walk, and when prompted will often walk on their hocks with assistance from their wings (Klasing, 2008).	No	n/a
Pantothenic Acid Deficiency	Apparent lameness and reluctance to walk, cracks and fissures prominent on feet (Klasing, 2008).	Yes	Brain: no reported lesions. Spinal Cord: myelin degeneration in all segments of the spinal cord (Phillips & Engel, 1939).
Nicotinic Acid Deficiency	Apparent lameness and reluctance to walk, with obvious enlargement of the hock joint and bowing of the legs (Klasing, 2008).	No	n/a
Pyridoxine (Vitamin B6) Deficiency	Abnormal gait characterised by 'jerky' leg movements, convulsions also apparent (Gries & Scott, 1972).	No	n/a
Biotin Deficiency	Apparent lameness and reluctance to walk, cracks and fissures prominent on feet (Klasing, 2008).	No	n/a
Folic Acid Deficiency	Reluctance to walk and apparent lameness in cases of severe chondrodystrophy (Klasing, 2008).	No	n/a
Cobalamin (Vitamin B12) Deficiency	Reluctance to walk and apparent lameness in cases of severe chondrodystrophy (Klasing, 2008).	No	n/a
Choline Deficiency	Reluctance to walk and apparent lameness in cases of severe chondrodystrophy (Klasing, 2008).	No	n/a
Calcium and Phosphorous Deficiency	Apparent lameness and reluctance to walk (Klasing, 2008).	No	n/a

Disease	Clinical features relevant to the CNS and gait	CNS pathology?	Brain & Spinal Cord histopathological features
Magnesium Deficiency	Profound lethargy, sometimes comatose, if disturbed can develop convulsions (Almquist, 1942).	No	n/a
Chlorine Deficiency	Short periods of paralysis with legs outstretched when startled, returning to normal over several minutes (Leach & Nesheim, 1963).	No	n/a
Potassium Deficiency	Paretic gait and reluctance to move, development of tetanic seizures (Klasing, 2008).	No	n/a
Manganese Deficiency	Chicks from manganese deficient parents can exhibit ataxia on excitement, and can often hold their head in abnormal positions (Caskey *et al.*, 1944).	No	n/a
Zinc Deficiency	Deficient birds exhibit enlarged hocks and chondrodystrophy, often developing an ataxic gait (Young *et al.*, 1958).	No	n/a
Other Diseases			
Dyschondroplasia	Reluctance to move, and stilted gait.	No	n/a
Valgus/Varus Deformity	Abnormal gait unilaterally or bilaterally, often reluctant to move.	No	n/a
Coxofemoral Degenerative Joint Disease	Lameness (unilateral or bilateral) and reluctance to move.	No	n/a
Spondylolisthesis (Kinky Back)	Persistent sitting on hocks with feet raised off the ground - completely unable to walk, will use wings in an attempt to locomote (Wise, 1975).	Yes	Brain: abnormal lesions not reported. Spinal Cord: lesions associated with spinal cord compression may be expected.
Defective Capital Femoral Ligament	Lameness and reluctance to move (Crespo & Shivaprasad, 2008).	No	n/a
Ruptured Gastrocnemius Tendon	Acute bilateral or unilateral lameness, affected bird typically sits on hocks with toes held flexed (Crespo & Shivaprasad, 2008).	No	n/a
Contact Dermatitis	Lameness and reluctance to move when lesions are associated with feet and hocks.	No	n/a
Hypoglycaemia-Spiking Mortality Syndrome of Broiler Chickens	Head tremors, apparent blindness, ataxia, and coma (Davis, 2008).	No	n/a

Disease	Clinical features relevant to the CNS and gait	CNS pathology?	Brain & Spinal Cord histopathological features
Mycotoxicosis	Ergotism can cause ataxia and inability to stand in experimental chicks (Bragg *et al.*, 1970). Fumonisins and aflatoxins have been reported associated with lameness (Prathapkumar *et al.*, 1997).	No	n/a
Toxicities			
Ionophore Toxicity	Weakness and reluctance to move, progressing to complete paralysis where bird lies sternally with neck and legs extended (Fulton, 2008).	No	n/a
Nicarbizin Toxicity	Dullness and ataxia (Bartov, 1989).	No	n/a
Dinitrotolumide (anti-coccidial) toxicity	Ataxia and torticollis (Jordan *et al.*, 1976).	No	n/a
Copper Toxicity	Weakness, convulsions and coma (Peckham, 1982)	No	n/a
Sodium (salt) Toxicity	In rare cases neurologic signs can occur, including prostration (Fulton, 2008).	No	n/a
Lead Toxicity	Ataxia, lameness and paralysis (Fulton, 2008).	Yes	Brain: focal areas of vascular damage in the cerebellum (Hunter & Wobeser, 1980). Spinal Cord: no reported lesions.
Organophosphorous Toxicity	Ataxia and prostration (Fulton, 2008).	Yes	Brain: no reported. Spinal Cord: Wallerian degeneration has been reported (Funk *et al.*, 1994).
Methionine toxicity	Toxicity due to DL-methionine has produced cervical paralysis in turkey poults (Hafez *et al.*, 1978).	No	n/a

2.5 Conclusion

The approach required to establish the causation of a new veterinary disease requires the use of both epidemiological and clinical techniques. Epidemiological analysis requires input from numerous stakeholders including farmers, veterinarians, laboratory technicians and researchers. Approach to epidemiological analyses commences broadly, taking into consideration all possible host, pathogen and environmental factors that may contribute to disease expression. Detailed on-farm epidemiological studies allow for further research to progress, such as attempts at experimental reproduction of a disease under investigation and analysis of the role of hypothesised causative or risk factors under controlled settings. The short turnaround time between batches of broiler chickens and the propensity for the industry to record on-farm data facilitates sound epidemiological studies.

Specific to diseases with neurological abnormalities, detailed neurological examinations of affected animals are essential in determining regions of the nervous system likely affected, which is essential for aetiological assessment. Neurological assessment in conjunction with adjunct diagnostics allows for a concise differential diagnosis list to be developed.

The aims of this study are as follows:

- To collate clinical and diagnostic information from broiler farms, field veterinarians and diagnostic laboratories in order to assist in defining the APS, describing its history and developing a differential diagnosis list;
- To conduct a detailed on-farm epidemiological study to identify and assess risk factors associated with cases of the APS;
- To attempt to reproduce the syndrome in a controlled experimental setting and subsequently:
    - Define a reliable reproduction model,
    - Define the syndrome as observed experimentally,
    - Further assess the role hypothesised causative factors play in the APS, and
    - Attempt to identify and isolate a causative agent;
- To propose mitigation strategies that can be implemented on-farm to reduce the economic and welfare costs associated with the APS; and
- To provide suitable understanding and direction for further research into the APS.

Chapter 3: General materials and methods

3.1 Isolator facility

All isolator experiments were conducted within the University of New England (UNE) isolator facility which accommodated 24 individual isolators (Figure 3.1). The facility was maintained under negative air pressure whilst the inside of each individual isolator was maintained under positive air pressure. Inflowing air was forced through a high efficiency particulate air (HEPA) filter and heated (depending on temperature settings) before being ducted into each isolator. Outflow air from each isolator unit was run through a series of scavenger ducts and through a HEPA filter prior to release outside of the facility. Complete control over air flowing through each isolator unit was maintained via a variable speed controller. There were between 12-23 air changes per hour in each isolator unit depending on the settings, which were varied based on chicken age. Additional heat within each isolator was provided via thermostatically controlled heat lamps.

Figure 3.1 UNE isolator facility: isolators were aligned into two rows of 12 each

The isolators were constructed with a stainless steel framework; however, the tops, sides and arm-pieces (gauntlets and gloves) were disposable plastic (Figure 3.2). The floors were constructed from 2.5 mm thick perforated stainless steel, with holes that were 12.7 mm in diameter. Of the total surface area of the floor, 49% was open. Stainless steel trays were placed over the perforated flooring in order to be able to maintain litter flooring (Figure 3.4). If litter flooring was used, the tray directly under the automatic watering system was perforated to enable waste water to flow through. The dimensions of each isolator unit were: width 66 cm, length 210 cm and height 88 cm.

Figure 3.2 View if individual isolator unit showing disposable sides and arm-pieces

The entire feed supply required for each isolator unit for the duration of the experiment was loaded and sealed into a feed hopper. The feed load was calculated such that chicken starter ration was available to chickens for the first 14 days of life and hence loaded into the hoppers first, followed by chicken grower ration which was available to the chickens as the starter ration was consumed. Water was supplied via a nipple watering system (Figure 3.3) connected to mains water supply. Additionally, for the first 2-3 days of chick placement, water stored within each unit was laid out in ice trays (Figure 3.3) and feed was laid out onto A3 sized paper.

Environmental enrichment was provided within each unit via four pieces of blue string hung from the ceiling to floor and autoclaved wood shavings placed in plastic trays to allow for dust bathing. In the case of the latter, enrichment litter trays were not provided in experiments when complete litter flooring was utilised.

Figure 3.3 View through two adjacent isolator units showing scratch trays, water set out in ice trays, nipple watering system, enrichment strings and perforated flooring

Figure 3.4 View through one isolator with complete litter flooring trays installed - the tray sitting under the watering system was perforated to allow waste water to flow through

3.2 Harvesting, handling and storage of challenge materials

Whole blood, spleen tissue, brain tissue and shed litter were used as challenge materials. Blood, spleen tissue and brain tissue were harvested from chickens (in the field or experimental) affected with the acute paralysis syndrome (APS). Litter was collected from sheds in the field in which the APS was occurring at the time. Challenge materials in the field were collected by a farm consulting veterinarian.

3.2.1 Collection and cryopreservation of whole blood

Whole blood used as challenge material was collected from a cutaneous ulnar vein into citrated vacuum tubes. If being sent to UNE from field cases, tubes were packed with ice blocks and freighted overnight to UNE and received within 24 hours of collection. Blood was pooled and dimethylsulphoxide (DMSO) (Sigma Life Science, St. Louis, MO, Lot: RNBC2918) was added at the rate of 10 % v/v. Blood was then transferred into 2 mL cryopreservation tubes (*Cryo.s*®, Greiner Bio One, Ref: 122263). The cryopreservation tubes were held in a cryopreservation container (*Mr. Frosty*® freezing container, Thermo Fisher Scientific Inc., North Ryde, NSW) overnight (approximately 12 -16 hours) at -80°C ensuring a freeze rate of no more than 1°C cooling per minute, before being transferred into liquid nitrogen. When required for use as challenge material, blood in cryopreservation tubes was thawed in a 37.5°C water bath and pooled prior to inoculation.

3.2.2 Collection, extraction and cryopreservation of spleen cells

Spleens collected from affected birds on post-mortem (PM) examination were rinsed with sterile phosphate-buffered saline (PBS) and stored in sterile specimen containers. If being sent to UNE from field cases, the sterile containers were packed with ice blocks and air freighted overnight to

UNE and received within 24 hours of collection. The following process was adopted in order to extract splenocytes for cryopreservation:

1. Prior to commencing, 50 mL of foetal bovine serum (HI FBS Gibco®, Invitrogen Corporation, Mulgrave, Victoria, Ref: 10100) and 7 mL of 100x antibiotic-antimycotic mix (Anti-Anti Gibco®, Invitrogen Corporation, Mulgrave, Victoria, Ref: 15240) (Penicillin G 10,000 units/mL, Streptomycin, 10,000µg/mL, Amphotericin B 25mg/mL) was added to one 500 mL bottle of cell culture medium (M199, Gibco® by Life Technologies, Mulgrave, Victoria, Ref: 11150). A 20 mL aliquot of this solution was removed and added to a 50 mL sterile tube and 5 mL of DMSO was added to make the cryopreservation solution.
2. A small amount of sterile PBS was added to a petri-dish, to which a spleen was added after capsule removal. The spleen was then homogenised with the PBS using sterile scissors and forceps initially, followed by the use of a 10 mL syringe and 18 g needle.
3. Homogenised spleen tissue was then put through a sterile standard tea strainer, followed by a sterile finer sieve.
4. Approximately 1 mL of filtrate was added to sterile cell culture tubes (Falcon®, BD Australia, North Ryde, NSW, Ref: 4-3033-2) pre-loaded with 3 mL of Ficoll-Paque Plus solution (GE Healthcare Biosciences, Sweden, Ref: 17-1440-02).
5. Cell culture tubes were then centrifuged at 450 × *g* for 15 minutes at 15°C.
6. The white cell layer was removed from each tube with a sterile transfer pipette, into separate sterile tubes containing 3 mL of sterile PBS. The tubes were then centrifuged at 450 x *g* for 15 minutes at 15°C to wash the white cells.
7. The supernatant was poured from each sterile tube and the cell layer from each transferred to a sterile 50 mL tube using a transfer pipette.
8. The volume in the 50 mL tube was made up to 8-10 mL by adding the cryopreservation solution described in step 1.
9. The complete solution containing splenocytes was then transferred into 2 mL cryopreservation tubes.
10. Cell quantification was performed using a Neubauer haemocytometer (Celeromics, Spain), following the manufacturer's instructions.
11. The cryopreservation tubes were then held in a cryopreservation container and placed into -80°C overnight (approximately 12 -16 hours) ensuring a freeze rate of no more than 1°C cooling per minute, before being stored in liquid nitrogen.

12. When required for use as challenge material, spleen cells were thawed in a 37.5°C water bath and pooled prior to inoculation.

3.2.3 Collection and cryopreservation of brain tissue

Whole brains collected from affected field birds on PM examination were rinsed in sterile PBS and stored in sterile specimen containers. They were then packed with ice blocks and freighted overnight to UNE and received within 24 hours of collection. On receipt at UNE, the process used for the extraction and cryopreservation of spleen cells (3.2.2) was used for brain tissue, excluding steps 4 - 5 (centrifugation in Ficoll-Paque Plus solution) and step 10 (cell quantification).

3.3 Enzyme-linked immunosorbent assay (ELISA) for Marek's disease virus in chicken sera

The ELISA used was that reported by Ralapanawe *et al.* (2015) which is an indirect ELISA useful for the detection of Marek's disease virus (MDV) specific antibodies. The MDV antigen is non-specifically adhered to each of a 96-well polyurethane plate. The method followed in performing this ELISA was:

1. Serum samples to be tested were diluted 1:100 with PBST (1,000 mL PBS pH7.4 + 0.5 mL Tween 20) containing 1 % skim milk powder and stored at 4°C until ready for use.
2. ELISA plates were coated with MDV antigen. 100 µL of a 1: 100 diluted (with 0.05 M carbonate-bicarbonate buffer pH 9.6) MDV antigen solution was added to each well. The MDV antigen solution was derived from Rispens CVI988 vaccine (Bioproperties Vaxsafe RIS®) and processed following the method reported by Ralapanawe *et al*. (2015).
3. After antigen coating, the plate was covered with plastic wrap and incubated at 4°C for a minimum of 16 hours.
4. After incubation, the liquid was removed and the plates were washed twice with PBST. For blocking, 100 µL of 1 % skim milk power in PBST was added to each. The plate was then covered with plastic wrap and incubated at room temperature for approximately one hour.
5. Following incubation, the blocking solution was removed and 100 µL of the diluted serum samples were added to allocated wells on each plate, followed by standard dilutions and blanks. A total of 10 standard dilutions were used with MDV antibody (derived from experiments in which SPF chickens were challenged with MDV) diluted with PBST at the following levels: 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, 1:4096, and

1:8192. The plates were then covered with plastic wrap and incubated at 37°C for one hour.

6. After incubation the contents of each plate were removed and washed twice with PBST. Then to each well, 100 µL of a 1: 5,000 dilution of anti-chicken IgY whole molecule peroxidase (Sigma, St. Louis, MO, ref: A9046) in PBST was added. Plates were then covered and incubated at room temperature for one hour.
7. After incubation the contents of each plate were removed and each plate was washed three times with PBST. Then to each well, 100 µL of substrate (100 mL citrate buffer pH 5.0 with 34 mg of o-phenylenediamine and 50 µL of 30 % w/v hydrogen peroxide) was added. Plates were then covered with aluminium foil and incubated at room temperature for 10 minutes.
8. Following the short incubation period, the reaction was stopped by adding 50 µL per well of 1 M sulphuric acid. The plates were then read in a microplate reader (Biorad Benchmark Microplate Reader, Hercules, CA) at 492 nm.
9. The threshold value for determining positive and negative samples was set at 569 units which was the highest titre derived from serum samples tested from five un-challenged specific pathogen free (SPF) chickens.

3.4 Molecular diagnostic methods

3.4.1 DNA extraction

DNA from approximately 25 mg of tissue (spleen or brain) or 200 µL of undiluted blood or spleen cell challenge material was extracted using the Isolate II Genomic DNA Kit (Bioline, Batch: GENO50/112G), following the manufacturer's instructions. Extracted DNA was quantified using a Nanodrop® ND-1000 IV-Vis spectrophotometer (Nanodrop® Technologies, Wilmington USA) and then stored at -20°C until required for analysis. If DNA concentrations measured > 100 ng/µL the DNA was diluted 1:10 with elution buffer (from Bioline kit).

3.4.2 RNA extraction

RNA from approximately 25 mg of tissue (spleen or brain) was extracted using the Isolate II RNA Mini Kit (Bioline, Batch: IS508-213E), following the manufacturer's instructions. Tissue which had been stored at -20°C from harvest was wrapped in aluminium foil and plunged into liquid nitrogen for 1 minute. This tissue was then homogenised in liquid nitrogen to a powder consistency using a mortar and pestle. Extracted RNA was quantified using a Nanodrop® ND-1000 IV-Vis spectrophotometer and stored at -80°C until required for analysis.

3.4.3 Standard polymerase chain reaction (PCR)

All PCR reactions contained 40 µM $MgCl_2$ (Fisher Biotec, Batch: 290622), 10 × reaction buffer (Fisher Biotec, Batch: 290112), 5 µM dNTP mix (Fisher Biotec, Batch: LA-290127), 120 ng forward primer, 120 ng reverse primer, 1.1 units Taq polymerase (Fisher Biotec, Batch: 281014) and 100 ng/µL or less of extracted DNA made to a total volume of 25 µL with sterile water. DNA amplifications were performed in a Bio-Rad C-1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules CA USA). The program used for all reactions was 94°C for 5 min, 60°C for 1 min, 72°C for 2 min; then 30 cycles of 94°C for 1.5 min, 60°C for 1 min, 72°C for 2 min; then 94°C for 1.5 min, 60°C for 1 min, 72°C for 2 min and 4°C for 5 min to complete the amplification reaction.

3.4.4 Reverse-transcriptase polymerase chain reaction (RT-PCR)

RT-PCR's were conducted using an Access RT-PCR Introductory System (Promega, Madison WI USA, Lot: 69690) following the manufacturer's instructions. The RT-PCR was carried out in a Bio-Rad C-1000 Touch Thermal Cycler and the selected program was as per the kit manufacturer's instructions.

3.4.5 Agarose-gel electrophoresis for analysis of standard PCR and RT-PCR products

Products from PCR reactions were analysed using agarose gel electrophoresis. Agarose powder (Promega, Madison USA, Lot: V3121) was dissolved in the Tris-acetate buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) to the required concentration, in a microwave oven for approximately 60 seconds. The molten gel was cooled to approximately 50°C on a mixing plate to which 0.04 µL/mL GelStar (BioWhittaker Molecular Applications, Rockland ME USA, Lot: 1316886) was added. The molten agarose solution was poured into a casting tray which was sealed with tape and had the well-comb in place. The gel was set for 20-30 minutes before 5 µL aliquots of the amplification reactions were mixed with 2 µL of DNA gel loading-dye (Life Techologies, Mulgrave VIC) and pipetted into allocated wells in the gel. Electrophoresis was performed in Tris-acetate buffer at 96 V for one hour. Gels were visualised under ultraviolet light using a BioRad Gel Doc EZ Imager (Biorad Laboratories, Hercules CA USA). The sizes of amplified DNA fragments were determined using a 100 bp DNA ladder (Promega, Madison WI USA, Lot: 69765).

3.4.6 Real-time polymerase chain reaction (qPCR)

Real-time PCR assays, specific for serotype-1 MDV (MDV1), herpesvirus of turkeys (HVT) and chicken anaemia virus (CAV), were used. The assays for MDV1 and HVT were developed and reported previously by Islam *et al.* (2004). The primer and probe targets for the MDV1 assay are based on the *meq* gene, whilst those for the HVT assay, target the *sorf*1 gene. The CAV assay was developed at UNE (Alsharari *et al.,* 2015) using primer sets reported by Zhang *et al.,* (2009).

Amplification reactions containing extracted and quantified DNA and mastermix were prepared using the liquid handling station CAS-1200 (Corbett, Sydney Australia). Each reaction contained 0.3 µM of each primer, 0.2 µM of the probe, 12.5 µL of qPCR supermix (Kapa Biosystems, Australia) and 5 µL of DNA template. Amplification reactions were performed in a Rotor-Gene Q thermocycler (Qiagen Pty Ltd., VIC Australia), using reaction conditions according to the manufacturer's instructions. Results for each assay were analysed using the Rotor-Gene Q software. A standard curve for each primer set was generated in each assay (Islam *et al.,* 2004) and subsequently used to determine a copy number of target sequences in unknown samples. For each assay, standard curves were generated using four standards covering a $10\text{-}10^5$ copy number range. All samples were randomised in each assay and samples were amplified in duplicate. Samples in which there was no amplification, or in which the amplification count was below the lowest standard, were treated as negative.

Chapter 4: Analysis of existing information on the acute paralysis syndrome and refinement of the differential diagnosis list

4.1 Introduction and background to the APS

An acute paralysis syndrome (APS) was first reported in March 2010 in a major chicken meat production region of Australia. The initial case was reported in mixed sex 32 day old Ross broiler chickens on one shed on farm 13. The APS was characterised by prostration and flaccid paralysis of the neck in affected chickens and elevated flock mortalities Diagnostic samples from this initial case were sent to the State Veterinary Diagnostic Laboratory (SVDL) (Menangle, NSW) for notifiable disease exclusion testing for ND and avian influenza (AI) and further diagnostics. From this initial testing, virulent ND and AI were excluded.

I visited the region from 21-24 October 2013 to obtain information pertaining to the history of the APS and husbandry practices used on broiler growing farms in this region. I was able to visit two farms which were experiencing cases of the APS: farm 1 which was experiencing the APS in 27 day old mixed sex broilers in one shed and farm 15 which was experiencing the APS in 35 day old mixed sex broilers in one shed.

There were a total of 18 broiler growing farms operating in this region and 14 of these farms (denominated farms 1-14) were located within an 8 km radius (201 km^2) of the feedmill supplying each farm (Figure 4.1). The plant which processed all chickens from the broiler farms in the region was located adjacent to the feedmill (Figure 4.1). Two farms (15 and 16) were located on a site approximately 68 km north-north-east of the feedmill and processing plant and an additional two farms (17 and 18) were located on a site approximately 57 km north-west of the feedmill and processing plant.

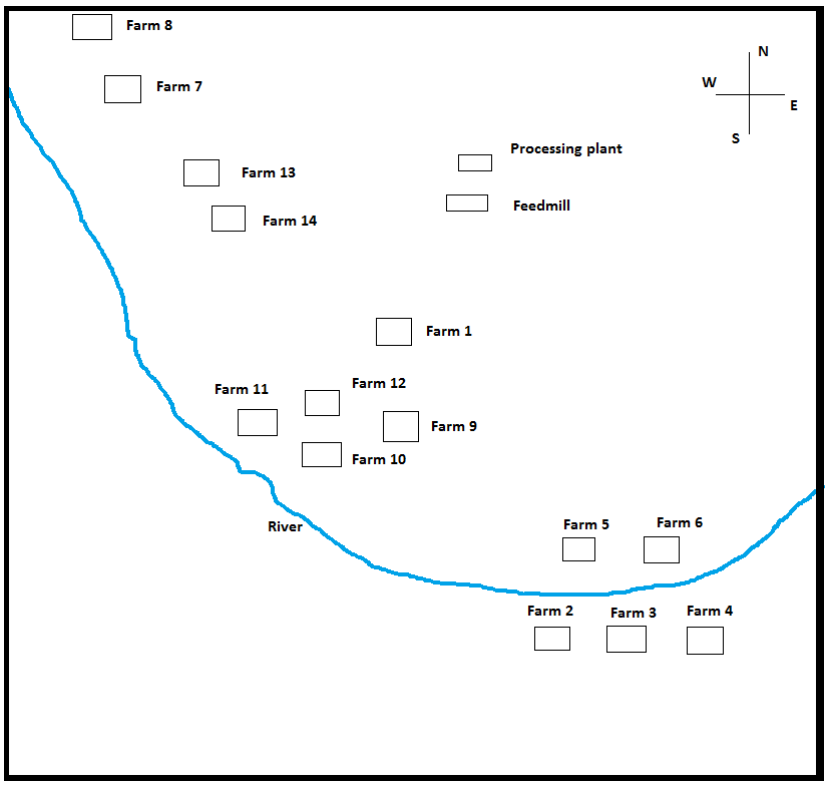

Figure 4.1 Approximate location of 14 of the broiler growing farms in proximity to the local feed mill and processing plant (sketch not to scale).

The breed Ross 308 was the sole breed grown on farms in the affected region. Parent flocks were typically vaccinated against Marek's disease (MD), infectious bronchitis (IB), coccidiosis, ND, *Salmonella typhimurium*, fowl pox, infectious laryngotracheitis, avian encephalomyelitis (AE), fowl adenovirus serotype 8 (FAV8), infectious bursal disease (IBD), CAV and egg drop syndrome 1976. The effectiveness of the vaccination regime used was monitored via serological testing at various ages of the parent flocks.

Broilers in the region were reared entirely inside through to a maximum of 56 days of age, with flocks being typically thinned-out for processing on four occasions from 34 – 56 days. The majority of broilers were hatched in a local hatchery. Smaller numbers of broilers were occasionally sourced from two other hatcheries within the state. Broilers were vaccinated for ND and IB at the hatcheries and received no further vaccination throughout their life.

Farms were single-age (each shed on a particular farm contained chickens of similar ages) and contained sheds of older (dirt floor) and/or or newer (concrete floor) design. Newer sheds were larger and thus accommodated more chickens than did the older sheds; however, all sheds had automated ventilation systems utilising fans, air in-flow vents and water pads as required. Shed litter was typically cleaned out and replaced after each batch of chickens in conjunction with thorough disinfection and formaldehyde fumigation of the sheds. All sheds had automated feeding

and watering (nipple) line systems. Rations were milled into pellet form by the local feed mill using mostly locally sourced ingredients which varied based on availability but consisted predominantly of wheat and sorghum.

This chapter sets out to define the syndrome based on its clinical appearance, to compile and evaluate on-farm and in-laboratory diagnostics that have been performed and to refine the differential diagnosis list as set out in Table 2.5.

4.2 Materials and methods

A case was defined as the presence of the APS in a single flock of broiler chickens, where a flock was considered as a single shed placement. Diagnostic information from cases of the APS was retrieved in the form of laboratory reports provided by farms and the SVDL. Data relating to serotype-1 MD virus (MDV1) load in shed dust and affected chicken tissues was obtained from the Poultry Health Research Group at the University of New England (UNE), Armidale NSW and subsequently collated. Data pertaining to haematology and biochemistry measurements were provided in laboratory report format by a consulting veterinarian and subsequently analysed using the program JMP 11 (SAS Institute, 2013) using a standard ANOVA model and a significance level of $P < 0.05$ was accepted for test statistics.

4.3 Results and diagnostic features of the APS

4.3.1 Clinical appearance of the APS

The typically affected broiler chicken was initially paretic and ataxic and demonstrated an increased tendency to sit, often with its head resting on the floor (Figure 4.2 – left panel). This progressed to a state whereby the affected chicken was rendered completely sternally recumbent, unable to stand or walk and was in a stuporous state. In this final stage of the APS, the head and neck of the affected broiler lay on the floor and there was marked flaccidity of the neck, such that if the head and neck were raised by the observer and released, the bird could not prevent its head and neck from falling rapidly to the floor again. The clinical appearance of affected chickens is shown in Figure 4.2.

Figure 4.2 Typical clinical manifestation of the APS in a 24 day old broiler on farm 1 (left panel) and two 35 day old broilers on farm 16 (right panel). Note in the right panel, the bird on the right demonstrated flaccid paralysis of the head on the neck, whereas the bird on the left could raise its head slightly on prompting. Photos: B. Sharpe.

Veterinarians and farm staff reported that the APS typically occurred at higher incidence in males and in larger birds in affected flocks. Additionally, they reported that affected chickens were typically pyretic and gross abnormalities were not typically detected. Morbidity rates were not specifically recorded.

4.3.2 Infectious disease diagnostics

Exclusion testing (utilising reverse-transcriptase polymerase chain reactions) for the notifiable diseases ND and AI were carried out on two cases of the APS in March 2010 and again in September 2013 through the SVDL. On both occasions testing failed to detect virulent ND virus or AI virus; however, in relation to ND virus a strain of low pathogenicity was detected on both occasions which was consistent with the vaccine virus used in the region.

Testing for the presence of *C. botulinum* toxins C and D was performed on two cases associated with this outbreak between 2010 and 2013. Serum samples from 10/10 affected chickens from one case and 6/6 affected chickens from a second case tested negative in the *C. botulinum* antigen type C and D ELISA (Thomas, 1991). At this point it was considered that with the negative serum ELISA results, botulism was unlikely the cause of the APS.

Results from shed dust testing for MDV1 load using quantitative polymerase chain reaction (qPCR) from 2010-2012 are shown in Figure 4.3. It was evident that high levels of MDV1 were present in shed dust up until July 2012 at which point vaccination of chickens with herpesvirus of turkeys (HVT) *in-ovo* at day 18 of incubation commenced. Following the implementation of HVT vaccination the MDV1 levels in shed dust declined sharply; however, cases of the APS continued to be observed with MD vaccination having no discernible effect on incidence. A total of 7/32 (22 %) spleen samples from unvaccinated chickens and 36/96 (38 %) of spleen samples from HVT vaccinated chickens were MDV1 positive between 2012 and 2013. One of three brain samples

from HVT unvaccinated chickens and one of five brain samples from HVT vaccinated chickens were MDV1 positive in 2012. All feather tips analysed in 2013 were from HVT vaccinated chickens and were negative for MDV1.

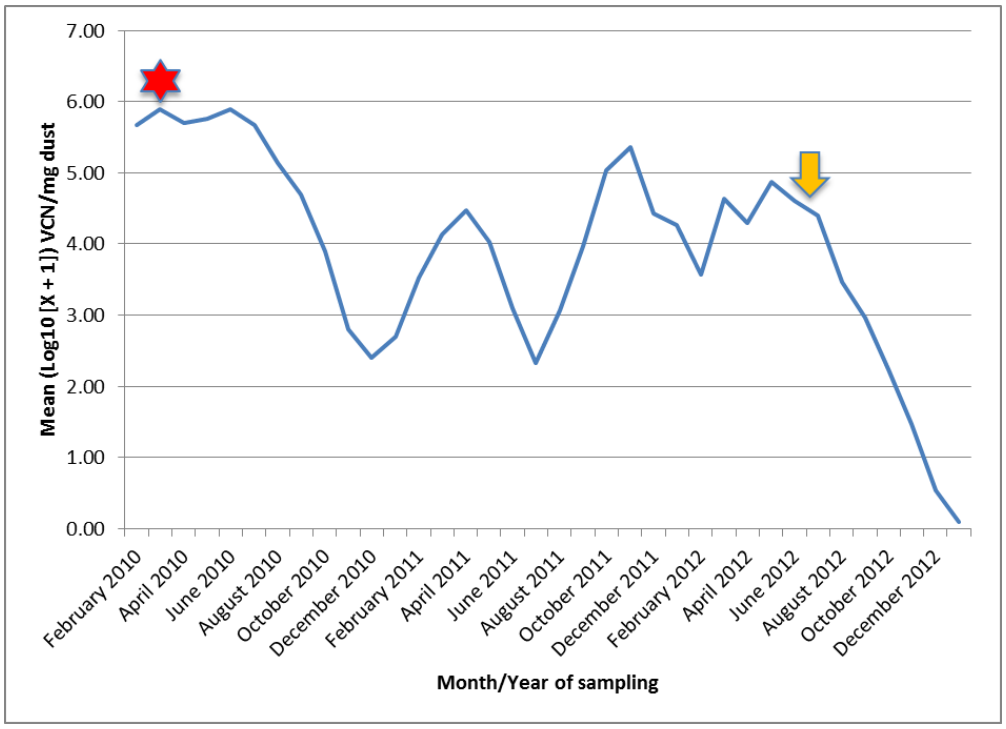

Figure 4.3 Mean MDV1 load (3-monthly rolling averages) in shed dust (n = 298) on broiler farms in the affected region 2010-2012. The red star indicates the point in time that the first case of APS was recorded and the yellow arrow indicates the point in time that HVT vaccination of broilers commenced.

4.3.3 Histological findings

During the period March 2010 – March 2014 an array of diagnostic pathology was performed at the SVDL at the request of consulting veterinarians. The main focus of such diagnostics was the CNS (brain and spinal cord) given the neurological signs clinically observed. Results from these investigations are summarised in Table 4.1.

4.3.3.1 Brain

Fixed brain sections from a total of 129 affected broiler chickens were evaluated. Inflammatory pathology was detected in 41 of these submissions. The characteristic findings in these sections were defined as vasculocentric encephalitis which included the perivascular cuffing of mononuclear cells often associated with endothelial hypertrophy and perivascular oedema and less commonly with infiltration of mononuclear cells into adjacent neuropil. The base of the cerebrum was the most consistent site of pathology. There was marked variation in the apparent severity of this pathology from subtle (Figure 4.4) to severe (Figure 4.5). Subsequent to the initial

discovery of this pathology in March 2010 and the clinical appearance of affected broilers, similar to that reported by Kenzy *et al.* (1973), Swayne *et al.* (1989abc), Witter *et al.* (1999) and Zander (1959), a tentative diagnosis of Marek's disease (MD) and more specifically the acute transient paralysis (TP) form of MD was made which lead to the implementation of HVT vaccination as previously mentioned.

4.3.3.2 Spinal Cord

Fixed spinal cords from a total of 54 affected broiler chickens were evaluated through January 2012 - March 2014. Most of these were cervical sections with a small number of lumbar sections examined (Table 4.1). Sections from a total of 20 of these chickens demonstrated Wallerian degeneration to a degree considered pathologic by the evaluating pathologists. Figure 4.6 shows two foci of marked Wallerian degeneration in a section of cervical spinal cord from a 50 day old broiler, characterised by chains of digestion chambers containing axonal debris. In some cases the Wallerian degeneration increased in severity further caudally in the examined sections. In one examined spinal cord section, mixed lymphoproliferative foci were noted in the dorsolateral and ventrolateral areas extending into the meninges.

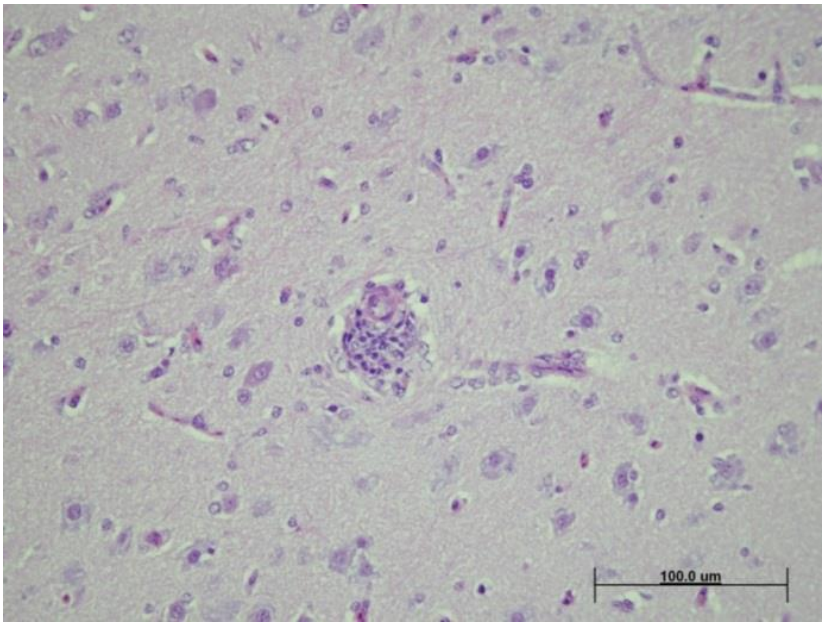

Figure 4.4 Base of the cerebrum from a 35 day old broiler affected with the APS, showing a small perivascular cuff of mononuclear cells surrounding a blood vessel (Photo: Rodney Reece).

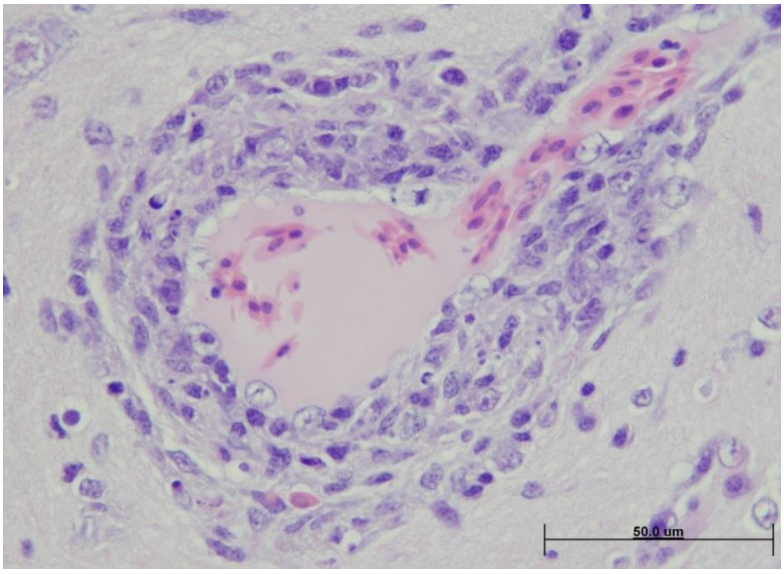

Figure 4.5 Base of the cerebrum from a 33 day old broiler affected with the APS, showing marked perivascular cuffing of lymphocytes and macrophages and associated endothelial hypertrophy and mild perivascular oedema (Photo: Rodney Reece).

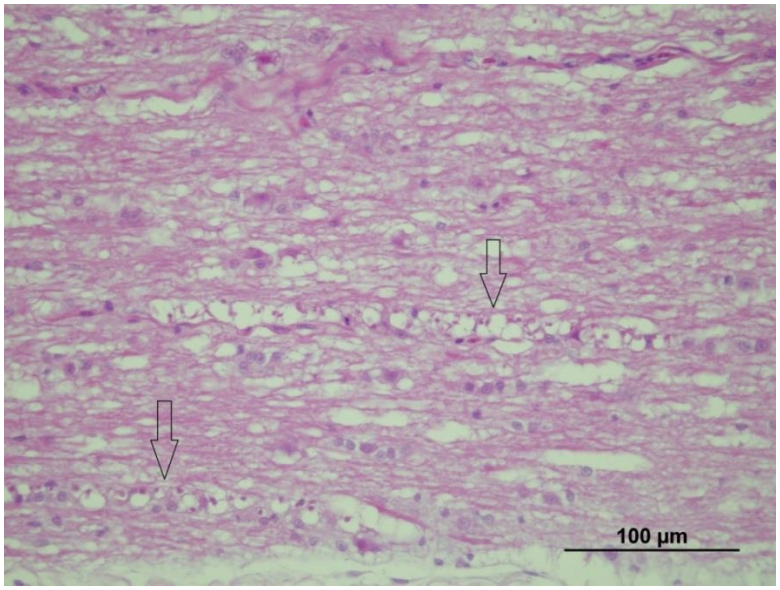

Figure 4.6 Cervical spinal cord (long section) from a 50 day old broiler showing chains of digestion chambers (arrows) containing axonal debris consistent with Wallerian degeneration (Photo: Rodney Reece).

4.3.3.3 Other organ systems

Lymphoproliferative lesions were detected associated with livers submitted from six cases as set out in laboratory report references (LRR) 5, 6, 7, 10, 11, and 12 (Table 4.1); spleens from three cases (LRR 5, 7, and 12) and bursae of Fabricius from 2 cases (LRR 6 and 36). In one case (LRR 16) evidence of infiltrative myelocytoma was detected in the liver, spleen, bursa of Fabricius, kidney and lung of one chicken which was considered consistent with avian leukosis.

Regressive lesions associated with the bursa of Fabricius which were considered consistent with endemic IBD infection were found associated with three cases (LRR 5, 22 and 36). Evidence of

transmissible proventriculitis was detected associated with the proventriculus of chickens in two cases (LRR 14 and 23). Liver pathology consistent with inclusion body hepatitis was found in one case (LRR 22) and hepatitis of uncertain aetiology in another case (LRR 24).

In one case (LRR 16) Wallerian degeneration in conjunction with perivascular inflammation was found associated with a sciatic nerve from one chicken and these findings were considered consistent with MD (type B lesion) by the evaluating pathologist. In another case (LRR 20) perivascular lymphoid foci were noted accompanying perineural vessels associated with a sciatic nerve in two chickens.

Coccidiosis was observed in two chickens from one case (LRR 26). Gastrocnemius tendon rupture was noted in one chicken in one case (LRR 15). Adductor muscle samples were submitted from 18 cases reported in Table 4.1 and pathology which may have been considered consistent with ionophore toxicity were not detected in any sample. Significant pathology was not detected associated with any other organ system as reported in Table 4.1.

The pathology associated with other organ systems reported in this section were considered to be incidental and not related to the APS given the small number of specific cases reported in relation to each finding across all cases of the APS.

Table 4.1 Summary of histological findings

Date	LRR	Farm	Age	# Birds	Brain lesions	Spinal cord lesions	Other findings
23-Mar-10	1	13	32d	5	Vasculocentric encephalitis, mostly in cerebrum in 3/5 samples	n/a	n/a
13-Dec-11	2	7	35d	2	NSF	n/a	n/a
19-Jan-12	3	14	35d	5	Vasculocentric encephalitis in 1 of 5 brains	n/a	n/a
24-Jan-12	4	14	43d	4	NSF	n/a	
27-Jan-12	5	8	28d	5	NSF	Wallerian degeneration in 1 of 5 birds	Lymphoproliferative lesions in livers (5/5) and spleens (5/5) consistent with MD, bursal histopathology consistent with IBD
27-Jan-12	6	8	28d	4	Vasculocentric encephalitis, mostly in cerebrum in 3/4 samples	n/a	Mild lymphoproliferative lesions in livers (3/4) and bursa's (4/4), bursa's display follicular depletion consistent with MD
9-Feb-12	7	7	49d	5	Vasculocentric encephalitis, mostly in cerebrum in 5/5 samples	n/a	Mild lymphoproliferative lesions in livers (5/5) and spleens (5/5) consistent with MD
16-Feb-12	8	8	51d	2	n/a	Mixed lymphoproliferative foci in dorsolateral and ventrolaternal area and involving meninges in both samples	n/a
16-Feb-12	9	12	33d	8	Vasculocentric encephalitis (mild) in 1 or 8 samples	n/a	n/a
29-Mar-12	10	7	23d	3	Vasculocentric encephalitis, centred in midbrain in 1/3 samples	NSF	Mild lymphoproliferative lesion in liver in 1 of 3 samples
28-Mar-12	11	14	33d	2	Mild vasculocentric encephalitis in 1/2 samples	NSF	Mild lymphocytic infiltration in 1/2 liver samples

Date	LRR	Farm	Age	# Birds	Brain lesions	Spinal cord lesions	Other findings
29-Mar-12	12	8	24d	2	NSF	n/a	No changes in adductor muscles to indicate ionophore toxicity, mild lymphoid proliferation in the liver and spleen of 1/2 birds
21-Jun-12	13	10	30d	1	Mild vasculocentric encephalitis	n/a	NSF in liver and bursa
20-Jul-12	14	14	21d	6	NSF	n/a	Evidence of transmissable proventriculitis in 1/6 birds
27-Jul-12	15	1	42d	3	NSF	n/a	Gastrocnemius tendon rupture in 1 bird
23-Aug-12	16	7	40d	1	Vasculocentric encephalitis	n/a	Multifocal infiltrative myelocytoma consistent with ALV subgroup J in multiple organs. Wallerian degeneration and perivascular inflammation in sciatic nerve consistent with early MDV
5-Oct-12	17	13	36d	2	NSF	n/a	Diffuse but mild lymphoproliferative enteritis in both birds
31-Oct-12	18	8	37d	2	NSF	n/a	NSF in other tissue
30-Oct-12	19	10	30d	2	Vasculocentric encephalitis in 1 of 2 brains	n/a	NSF in other tissue
21-Dec-12	20	14	39d	9	Vasculocentric encephalitis in 7 of 9 brains	n/a	Perivascular lymphoid foci in 2/9 sciatic nerves
9-Jan-13	21	8	41d	4	NSF	n/a	NSF on sciatic nerves
10-Jan-13	22	12	34d	6	Mild vasculocentric encephalitis in 1 of 6 brains	n/a	Evidence of IBD in 2 of 6 birds, inclusion body hepatitis in 1 of 6 birds
9-Jan-14	23	10	34d	5	Vasculocentric encephalitis in 3 of 5 brain submissions	n/a	Lesions consistent with transmissable viral provetrculitis in 2 of 5 birds
24-Jan-13	24	10	49d	1	NSF	n/a	Evidence of hepatitis, unknown aetiology

Date	LRR	Farm	Age	# Birds	Brain lesions	Spinal cord lesions	Other findings
24-Jan-14	25	n/a	47d	3	NSF	n/a	NSF
25-Jun-13	26	13	28d	2	Vasculocentric encephalitis in both specimens	n/a	Coccidiosis present, E. acervulinum
25-Jul-13	27	7	48d	4	NSF	n/a	NSF
13-Feb-13	28	10	7d	1	NSF	n/a	n/a
4-Sep-13	29	13	33d	15	Mild vasculocentric encephalitis in 3 of 15 brains	Wallerian degeneration present in 1/1 spinal cord, no inflammatory infiltrate	NDV qPCR negative from cloacal swab x 3, 1 sample NDV pos - V-4 type, influenza A matrix PCR negative from cloacal swab x4.
19-Sep-13	30	9	24d	4	NSF	Wallerian degeneration present in 3/4 cervical spinal cords examined	NSF
18-Sep-13	31	13	50d	2	NSF	Wallerian degeneration present in 2/2 cervical spinal cords examined	NSF
18-Sep-13	32	7	41d	3	NSF	Wallerian degeneration present in 3/3 cervical spinal cords examined	NSF
10-Oct-13	33	3	28d	5	Vasculocentric encephalitis of cerebrum in 2/5 brains sections examined	Wallerian degeneration present in 4/5 cervical spinal cords examined	NSF
17-Oct-13	34	15	28d	3	Vasculocentric encephalitis of cerebrum in 3/3 brain sections examined	n/a	n/a
6-Nov-13	35	13	33d	3	NSF	n/a	n/a
7-Nov-13	36	1	37d	3	Vasculocentric encephalitis of cerebrum in 2/3 brain sections examined	Wallerian degeneration present in 3/3 cervical and lumbar spinal cords examined	Lymphoproliferative lesions in 1/3 bursa's. Bursa's show evidence of endemic IBD
15-Nov-13	37	7	30d	6	n/a	n/a	Clostridium botulinum toxin antigen ELISA negative for 6/6 serum samples
20-Mar-14	38	1	40d	1	NSF	Wallerian degeneration evident in cervical and lumbar cord.	Lymphoproliferative lesion detected in the spleen, pancreas and adrenal gland. Atrophy detected in thymus and bursa, consistent with Marek's disease.

LRR: laboratory report reference, **NSF:** no significant findings, **n/a:** relevant samples or information not submitted.

4.3.4 Complete blood cell counts and biochemistry analyses

In one flock with the APS complete blood cell counts were performed from five clinically affected and five clinically normal chickens. Blood was collected by the attending veterinarian and testing was outsourced to Idexx Laboratories, Rydalmere NSW. Results were provided to me in laboratory report format and these results in conjunction with my analyses are provided in Table 4.2. Heterophilia was noted in all 10 samples, the mean heterophil count ($x10^9$ cells) for paralysed chickens was 18.5±3.2 and for normal chickens 11.5±3.2, the difference between the two was non-significant (P = 0.16). Monocytosis was evident in three samples from paralysed chickens and two samples from normal chickens, the difference between mean monocyte counts from paralysed and normal chickens was non-significant (P = 0.39). Basophilia was present in all samples from paralysed chickens and three samples from normal chickens and the difference in mean basophil counts between paralysed and normal chickens was non-significant (P = 0.92). Lymphopaenia was detected in all samples from paralysed chickens and three samples from normal chickens. The average lymphocyte count ($x10^9$ cells) of paralysed chickens was 1.7±1.9 and for normal chickens 8.9±1.9 and the difference between the two was significant (P = 0.03). The lymphopaenia was considered most likely resultant from stress.

Table 4.2 Summary of haematological analyses from 10 chickens from one flock

Sample number	Paralysis (Y/N)	PCV (L/L)	WBC ($x10^3$)	Het. ($x10^9$)	Lymph. ($x10^9$)	Mon. ($x10^9$)	Eos. ($x10^9$)	Baso. ($x10^9$)	Plasma protein (g/L)
Reference range		0.24-0.43	12.0-30.0	3.0-6.0	7.0-17.5	0.1-2.0	<0.9	<0.9	
1	Y	0.3	26.1	18.3	1.8	4.2	0	1.8	43
2	Y	0.33	9.9	7.5	0.8	0.2	0	1.4	40
3	Y	0.35	38.2	27.9	1.5	6.9	0	1.9	50
4	Y	0.32	18.7	14.4	1.9	1.3	0	1.1	43
5	Y	0.3	34.7	24.6	2.4	3.5	0	4.2	47
6	N	0.3	13.6	6.4	5.6	1.2	0	0.4	43
7	N	0.33	16.2	7	4.2	2.1	0	2.9	40
8	N	0.33	34.7	18.7	8.7	4.2	0	3.1	45
9	N	0.31	29.1	17.2	6.7	1.7	0	3.5	43
10	N	0.32	29.5	8.3	19.2	0.9	1.2	0	45
Mean (paralysed birds)		0.32±0.01	25.52±4.67	18.5±3.2	1.7±1.9	3.22±0.92	0	2.08±0.65	44.6±1.4
Mean (normal birds)		0.32±0.01	24.62±4.67	11.5±3.2	8.9±1.9	2.02±0.92	0.24±0.17	1.98±0.65	43.2±1.4
Effect of paralysis (P=)		0.86	0.89	0.16	**0.03**	0.39	0.35	0.92	0.50

PCV = packed cell volume, WBC = white blood cells, Het = heterophils, Lymph = lymphocytes, Mon = monocytes, Eos = eosinophils, Baso = basophils.

From the same chickens as reported in Table 4.2 above, a selection of biochemistry and blood-gas measurements were performed by the attending veterinarian using a iStat® handheld biochemistry analyser (Abbott Point of Care, Illinois USA) and these results are reported in Table 4.3. Significant differences were detected in plasma sodium (Na) (P = 0.01) and potassium (K) (P = 0.02) levels between paralysed and clinically normal birds. The mean plasma Na level for

paralysed chickens was 150.2±1.0 mmol/L and for clinically normal chickens 145.6±1.0 mmol/L; however, both measurements fell within the reference range for broilers reported by Bowes *et al.* (1989) being 145.5 – 150.5 mmol/L. The mean plasma K level for paralysed chickens was 4.20±0.18 mmol/L and for clinically normal chickens 4.96±0.18 mmol/L. The reference for plasma K in broilers as reported by Bowes *et al.* (1989) is 5.0-7.0 mmol/L. The mean value for clinically normal chickens was just below the bottom end of the reference range whereas the result from paralysed chickens was well below the reference range.

Table 4.3 Summary of biochemical and blood gas measurements from 10 chickens from one flock

Sample number	APS (Y/N)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	Glucose (mmol/L)	TCO_2	pH	PCO_2	HCO_3 (mmol/L)	Base excess	Anion Gap
1	Y	149	4.8	115	12.6	28	7.463	37.6	27.0	3	12
2	Y	149	4.2	113	10.7	30	7.399	46.9	29.0	4	12
3	Y	149	4.2	111	12.9	33	7.462	44.7	32.0	8	10
4	Y	155	3.6	118	12	32	7.487	40.5	30.7	7	10
5	Y	149	4.2	115	13.1	27	7.424	39	25.5	1	13
6	N	146	4.6	109	12.5	32	7.540	35.9	30.6	8	11
7	N	148	5.6	115	12.1	29	7.393	44.8	27.3	2	11
8	N	146	5	112	11.6	26	7.417	38.2	24.6	0	14
9	N	144	4.8	113	12.7	24	7.416	35.6	22.9	-2	14
10	N	144	4.8	110	11.8	32	7.524	37.4	30.8	8	8
Mean (paralysed birds)		150.2±1.0	4.20±0.18	114.40± 1.12	12.260± 0.339	30.0±1.4	7.44701± 0.0243	41.7±1.7	28.84±1.39	4.60±1.72	11.400 ± 0.900
Mean (normal birds)		145.6±1.0	4.96±0.18	111.80± 1.12	12.140± 0.339	28.6±1.4	7.4580± 0.0243	38.4±1.7	27.24±1.39	3.20±1.72	11.600 ± 0.900
Effect of paralysis (P=)		**0.01**	**0.02**	0.14	0.81	0.50	0.76	0.20	0.44	0.58	0.88

APS = acute paralysis syndrome, NA = sodium, K = potassium, Cl = chlorine, TCO_2 = total carbon diozide, PCO_2 = partial pressure of peripheral carbon dioxide, HCO_3 = bicarbonate

4.3.5 Neurological evaluation of affected chickens

On my visit to the affected region in 2013, 10 broiler chickens affected by the APS were examined over two farms (five from each). A neurological examination of each affected chicken was performed following the methodology set out in Chapter 2. The neurological examination findings were consistent in all of these chickens and are summarised in Table 4.4 which outlines the steps followed in each neurological examination, key findings at each step and finally a summary as to whether findings were normal or abnormal at each step. The summary of these results are as follows:

- Chickens had altered mental states;
- Chickens had abnormalities of gait;
- Cranial nerve function was intact in all chickens;
- Proprioceptive ability was intact in all chickens;
- Spinal reflexes associated with wings, legs and vent were normal in all chickens; and

- Nociception was intact in all chickens.

Based on the neurological examinations, the likely site of lesions in the CNS of affected birds was determined to be within the brain (Chapter 2). Given the normal functioning of cranial nerves in affected chickens this further localised lesions to within the forebrain (cerebrum and/or diencephalon). Whilst flaccidity of the neck could be explained by a cervical spinal lesion, such was not consistent with the lack of UMN signs associated with the wings, legs and vent.

Table 4.4 Summary of findings from neurological examinations from 10 affected chickens

Step in examination		Findings	Normal (Yes/No)
1.	**Observation**	-Change in mentation as chickens progressed from obtundation to stuporous -Gait was ataxic and combined with a wide-based stance early on in the syndrome but was completely absent at the final stage of the syndrome at which point affected chickens could not stand -Affected chickens retained the ability to vocalise -Chickens affected at the early stage were reluctant or unable to stand and walk but could hold their heads in a normal position, however chickens in the final stage of the syndrome lost their ability to hold their heads and necks off the ground.	**No**
2.	**Palpation**	-No evidence of asymmetry, masses or tenderness resultant from palpation of the muscles and skeleton.	**Yes**
3.	**Cranial nerves**	-Olfactory: not tested (no test applicable) -Optic: menace response intact, pupillary light reflex not tested -Oculomotor: eyeball position normal, menace response intact, pupillary light response not tested -Trochlear: eyeball position normal -Trigeminal: palpebral reflex present and beak tone present -Abducens: eye ball position normal -Facial: not tested (no test applicable) -Vestibulocochlear: chickens responded to startle mildly -Glossopharyngeal: chickens did not regurgitate despite heads being down on ground so gag reflex intact -Vagus: gag reflex intact -Accessory: not tested (no test applicable) -Hypoglossal: tongue function appeared normal.	**n/a** **Yes** **Yes** **Yes** **Yes** **Yes** **n/a** **Yes** **Yes** **Yes** **n/a** **Yes**
4.	**Postural reactions**	-Chickens maintained proprioceptive ability at all stages of the syndrome as they could return their wings to a folded position and return their legs and feet beneath their bodies if moved.	**Yes**
5.	**Spinal reflexes**	-Vent-sphincter reflex: pinching of the vent of affected chickens resulted in contraction of the vent and movement of the tail confirming this reflex was intact, thus caudal segments of the spinal cord were intact -Pedal-flexor reflex: withdrawal reflex present bilaterally thus ischiatic and lumbosacral plexus intact. Crossed extensor reflex absent -Patellar reflex: not tested -Wing withdrawal reflex: withdrawal reflex present bilaterally and crossed extensor reflex absent, thus brachial plexus intact.	**Yes** **Yes** **n/a** **Yes**
6.	**Pain perception**	-Chickens actively attempt to remove leg on application of painful stimuli to toes.	**Yes**

4.4 Discussion

In the early stages of the APS when chickens developed an ataxic gait and increased tendency to sit, it was impossible to distinguish the APS from other causes of gait abnormalities. Thus the broad differential diagnosis list for the APS presented in Table 2.5 encompassed all causes of gait deficits and neurological disease. It was the progression from the early stages of the APS to a state in which affected chickens were completely paralysed, rendered sternally recumbent and when flaccid paralysis of the neck was observed that identified the APS.

The management and husbandry standards employed on broiler growing farms in the region affected with the APS were considered to be of high standard and there were not any obvious problems in relation to husbandry that were considered as risk factors to the APS from gross observation on my visit to the region.

The detection of a low pathogenicity ND virus in affected chickens on two occasions was consistent with chickens being vaccinated with ND virus at hatch. A paper by Nakamura *et al.* (2008) reported three cases of severe non-purulent encephalitis in broiler chickens aged 25-46 days subsequent to ND vaccination. Neurological signs included depression, somnolence and leg paralysis. Additional findings in affected chickens in these three cases included necrotising pancreatitis characterised by white spots on the pancreas, discolouration and enlargement of the kidneys and spleen and respiratory signs. Brain histopathology of affected chickens included perivascular cuffing, neuronal degeneration, necrosis and gliosis in the cerebrum, cerebellum and medulla oblongata. The neurological presentation as described by Nakamura *et al.* (2008) is somewhat similar to chickens affected with the APS. However, they did not describe the flaccid paralysis of the neck as seen in chickens affected with the APS and, in the APS, the histopathology tended to be more inflammatory, less degenerative and was typically restricted to the cerebrum. This in addition to the consistent lack of respiratory and other signs observed in chickens affected with the APS and the irregular occurrence of the APS in flocks despite ND vaccination being consistent clearly separates the APS from the syndrome described by Nakamura *et al.* (2008).

The flaccid paralysis associated with botulism is similar to the APS in relation to the neck; however, chickens affected with botulism also typically demonstrate flaccid paralysis of the wings, legs and eyelids all of which were not observed in broilers with the APS. Additionally, the inflammatory CNS pathology observed associated with the APS is not observed in cases of botulism. The ELISA used to detect botulinum toxin in serum samples had sensitivity of 70% and specificity of 96% (Thomas, 1991) and is considered less sensitive than the mouse inoculation

bioassay (Thomas, 1991). Given this, although the ELISA failed to detect botulinum toxin in the serum of any affected chicken tested, these negative results cannot be used alone to exclude botulism from the differential diagnosis list. A mouse bioassay was not attempted to assess for the presence of botulinum toxin in serum, despite it being the most sensitive and reliable method (Dohms, 1987; Thomas, 1991). *C. botulinum* toxin was considered unlikely to be the cause of the APS due to the lack of wing, leg and eyelid paralysis in chickens affected with the APS, the presence of inflammatory CNS pathology in chickens affected with the APS and the negative toxin ELISA results from serum samples tested from affected chickens.

As described in 4.3.3, brain histopathology was only detected in 32% of brains sections evaluated from affected chickens. There are three possible reasons as to why this figure is relatively low. Firstly, the lesions were often subtle and thus could have been easily missed in sectioning of samples. Secondly, post-mortem degeneration could have masked the inflammatory lesions (particularly if subtle) and such degeneration was marked in cases when chickens were submitted to the laboratory chilled or there was an extended period between sampling and fixation of samples. Thirdly, in each case only a small proportion of brain was actually examined. Wajid (2013) also described similar inflammatory brain pathology and additionally malacia and haemorrhage associated with an earlier case of the APS, which were not findings described by SVDL pathologists.

In considering the gross appearance of chickens affected with the APS and inflammatory brain histopathology, a tentative diagnosis of MD and specifically the acute TP form was made by veterinarians and the SVDL pathologist. Additionally supporting this tentative diagnosis was the high level of MDV1 in shed dust in the region (Figure 4.3) and work by Wajid (2013) which demonstrated the presence of MDV1 by qPCR in 3/3 spleens collected from affected chickens in 2010. HVT vaccination was implemented (*in ovo* day 18 of incubation) in July/August 2012 (Figure 4.2) and despite this the APS continued to occur. If it was indeed MDV1 causing the APS, the continued presence of the APS after HVT vaccination could be explained if the MDV1 was a variant and HVT did not induce effective protection. Such cases are well documented (Eidson *et al.* 1981; Witter *et al.*, 1980). Wajid (2013) isolated MDV1 DNA from one dust sample from farm 13 in 2010 prior to the introduction of the HVT vaccination regime and amplified the *meq* gene for sequencing. It was found that the *meq* gene in the isolate aligned with that of the MDV1 isolate Md5 reported by Tulman *et al.* (2000), however with four relative single nucleotide polymorphisms (SNP's) and three SNP's relative to other Australian isolates (Wajid, 2013).

As is evident in Figure 4.2, levels of MDV1 in shed dust from meat chicken farms in the region declined sharply subsequent to HVT vaccination, which would be inconsistent with MDV1 inducing the APS given its continued presence. Attempts at detecting MDV1 from HVT vaccinated chickens affected with the APS was performed with 36/96 (38 %) of spleen samples, 1/5 of brain samples and 0/32 feather tips being MDV1 positive. It is unlikely that MDV1 is the cause of the APS given the elimination of MDV1 from shed dust in the region and only a small proportion of affected chicken samples testing positive for MDV1 since HVT vaccination. However, with a proportion of affected and HVT vaccinated chickens testing positive for MDV1, it cannot be ruled out entirely at this stage. This provides direction for future work as the absence of MDV1 in tissues from vaccinated chickens affected with the APS from controlled experiments in conjunction with the MDV1 laboratory data presented herein would be suitable grounds for ruling out MDV1 as a differential diagnosis for the APS.

Degenerative histopathology was observed in 37% of spinal cord submissions. Wallerian degeneration can result from axonal damage and one likely cause is from inflammation within the spinal cord which induces pressure trauma and necrosis. Inflammatory pathology of the spinal cord is often a feature of cases of MD, AE, ND and *Escherichia coli* induced osteomyelitis/synovitis. Evidence of other causes of spinal cord compression, such as anatomical abnormalities present in cases of spondylolisthesis, have not been found in chickens affected with the APS. Wallerian degeneration in the spinal cord has been reported associated with experimental induction of organophosphate (OP) toxicity (Funk *et al.,* 1994); however, the inflammatory brain and spinal cord pathology has not been reported to be associated with OP toxicity and in the clinical syndrome associated with OP toxicity, neck flaccidity is not a feature (Fulton, 2008). The spinal cord pathology observed was not consistent with the normal spinal reflexes observed in affected chickens, which may suggest that the Wallerian degeneration observed was not pathological, rather incidental.

The pathology associated with other organ systems reported in 4.3.3.3 were considered to be incidental and not related to the APS given the small number of specific cases reported in relation to each finding across all cases of the APS. Wajid (2013) reported early lymphomatous foci consistent with MDV infection associated with the kidney, liver and lungs from a portion of affected chickens sampled from a case of the APS in June 2010. These findings are consistent with the high MDV1 load in shed dust at the time (Figure 4.3). Additional pathological findings by Wajid (2013) were subcapsular liver haemorrhage (60%) and congestion in the kidneys (17%) and lungs (20%) in affected chickens, which were not reported findings of the SVDL pathologists and are likely incidental to the APS.

Clinical pathology was only performed from one case which unfortunately limits any interpretations that can be drawn. The observed leukograms of affected chickens were suggestive of stress which was an unsurprising finding given the clinical syndrome. Biochemical abnormalities of affected chickens relative to normal chickens were hypernatraemia and hypokalaemia, which were considered consistent with dehydration and/or pyrexia. Dehydration could be expected in recumbent chickens given that their access to water would have been restricted. Pyrexia could be explained in affected chickens if the syndrome was due in part to the presence of an infectious agent. Farm staff and veterinarians have observed elevated body temperatures in affected chickens which could be from actual pyrexia, associated with which a relative leukocytosis would be expected, or alternatively an inability of affected chickens to dissipate body heat due to recumbency. Severe hypokalaemia can induce flaccid paralysis in humans and other species; however, it was thought that the degree of hypokalaemia in affected chickens was not severe enough to account for the flaccid paralysis in affected chickens, as in humans circulating potassium levels of < 2.5 mmol/L induce flaccid paralysis (Vicart *et al.*, 2014). While these blood cell counts and biochemistry findings are a useful adjunct in attempting to understand the pathological processes that are occurring with the APS, greater sample sizes over multiple cases of the APS are necessary to confirm the abnormalities described.

There are a large number of diseases that are associated with neurological deficits and/or gait abnormalities in conjunction with CNS histopathology, including: MD, AE, ND, highly pathogenic AI, arbovirus infection, avian leukosis, systemic salmonellosis, systemic *E. coli,* fowl cholera, listeriosis, mycotic encephalitis, toxoplasmosis, avian malaria, vitamin E deficiency and lead toxicity (Table 2.5). The histopathological lesions as observed in the brain from broilers with APS typify a non-granulomatous and non-purulent encephalitis or vasculocentric encephalitis, dominated by mononuclear cells. Whilst inflammatory CNS histopathology can be observed in affected chickens with systemic salmonellosis, systemic *E.coli,* listeriosis or toxoplasmosis (Table 2.5), the implication of each of these agents in the APS is less likely because of the repetitive absence of bacterial colonies in examined CNS sections from broilers with APS which is inconsistent with systemic bacterial infection, the consistent lack of gross post-mortem findings in broilers affected with the APS typical of systemic bacterial infection, the consistent lack of micro-abscessation in CNS sections from broilers with APS typical of chickens affected by listeriosis and similarly the absence of cysts associated with the CNS of broilers affected with the APS typical of toxoplasmosis. Based on the typical pattern of lesions from the above mentioned list; MD, AE, ND, highly pathogenic AI and arboviral infection are most similar to the APS based on the presence of the inflammatory CNS histopathology associated with each. Table 4.5 summarises

the neurological presentation and observed CNS pathology of the APS in comparison with these five most similar infectious diseases based on CNS pathology. The neurological presentation with paralysis of the neck and stupor being the most obvious signs is most similar to the TP form of MD. Brain and spinal cord histopathology observed in chickens affected with APS shares features with the other diseases listed; however, the restriction of vasculitis to the cerebrum and the presence of Wallerian degeneration in the spinal cord are unique features. Investigations reported herein have ruled out ND and AI as differential diagnoses for the APS.

Table 4.5 Comparison of the neurological presentation and CNS pathology between the APS and viral diseases of similar presentation

Disease	Neurological presentation	Brain histopathology	Spinal cord histopathology
APS	Initial paretic and wide-based gait progressing to stupor and flaccid paralysis of the neck	Perivascular cuffing of mononuclear cells and often progressing to vasculitis with perivascular oedema and endothelial hypertrophy in the cerebrum	As for the brain and in conjunction with wallerian degeneration characterised by the presence of chains of digestion chambers, swollen axons, oligodendroglial cells and degenerate macrophages
MD (TP)	Varying degrees of ataxia, flaccid paralysis neck and/or limbs	Vasculitis with obvious perivascular oedema and vacuolation though the cerebrum, midbrain, brainstem and cerebellum	As for brain, lesions in the spinal cord less commonly reported
AE	Progressive ataxia, increased tendency to sit, tremors of the head and neck, prostration in young chicks (< 3 weeks of age)	Non-purulent encephalomyelitis in all regions of the brain	As for brain
ND	Listlessness, weakness, prostration, muscle tremors, torticollis, opisthotonus, paralysis of the wings and legs	Non-purulent encephalomyelitis, neuronal degeneneration and of the midbrain, brainstem and cerebellum	As for brain
AI	Depression, tremors of the head and neck, inability to stand, torticollis, opisthotonus, unusual position of the wings and legs	Lymphocytic meningoencephalitis, neuronal necrosis, gliosis and haemorrhage in all regions of the brain	As for brain, lesions in the spinal cord less commonly reported
Arbovirus infection (e.g. WNV)	Tremor and paralysis	Non-suppurative encephalitis	Not reported in chickens

APS = acute paralysis syndrome, MD = Marek's disease, TP = transient paralysis, AE = avian encephalomyelitis, ND = Newcastle disease, AI = avian influenza, WNV = West Nile virus.

In classical MD the presence of perivascular cuffing in the brain is often accompanied by focal areas of gliosis (Payne *et al.,* 1976). The latter was not a typical feature observed associated with

chickens affected with the APS. The TP form of MD is typically characterised by perivascular cuffing and evidence of progression to vasculitis and vacuolation in the brain most prominently in the cerebellum of affected chickens (Swayne *et al.,* 1989abc), as opposed to relative confinement to the cerebrum in cases of APS. The histopathological lesions as observed in TP are similar to what has been observed in chickens affected with the APS; however, the absence of lesions in the cerebellum of chickens affected with the APS and the relative lack of vacuolation are obvious points of difference. Wallerian degeneration as noted in spinal cord sections from chickens affected with the APS is not consistent with paralysis in any form of MD.

In AE, the presence of perivascular cuffing of mononuclear cells and areas of microgliosis are commonly found throughout the brain and spinal cord (Calnek, 2008), the former being evident in combination in some sections observed from affected chickens in this experiment. In ND and highly pathogenic AI perivascular cuffing is often seen in the CNS of affected chickens in addition to focal gliosis, endothelial hypertrophy and neuronal degeneration (Alexander & Senne, 2008; Swayne & Halvorson, 2008), somewhat similar to findings in this case. However, evidence of neuronal degeneration in the APS was restricted to the spinal cord thus being an obvious point of difference between ND and AI and the APS.

In some arboviral infections of chickens, such as West Nile virus (WNV), perivascular cuffing of mononuclear cells has been noted in conjunction with areas of obvious necrosis (Senne *et al.*, 2000). Necrotic areas were not evident in brains from APS affected chickens, thus being an obvious point of difference to WNV disease. Of the 12 families of arboviruses, only viruses of the *Togaviridae* and *Flaviviridae* families have been known to affect domestic poultry (Guy & Malkinson, 2008). In the *Togaviridae* family these include eastern equine encephalitis virus which primarily affects pheasants, western equine encephalitis virus, which has been mostly associated with turkeys and highlands-J virus which has been most associated with chukar partridges and turkeys (Guy & Malkinson, 2008). In the *Flaviviridae* family these include WNV which primarily affects geese and Israel turkey meningoencephalitis virus which affects turkeys (Guy & Malkinson, 2008). Recently in Australia, Kunjin virus (a WNV subtype) has induced neurological disease in equine populations (Frost *et al.,* 2012); however, arboviral induced neurological disease has not been reported in Australian poultry populations. Neurological disease of meat and layer poultry caused by arboviruses in the field are scarcely reported; however, WNV has been shown capable of inducing non-suppurative encephalitis in chickens after experimental inoculation (Senne *et al.,* 2000). Arboviruses rely on an arthropod vector for transmission and as a result are often seasonal in incidence. In the region affected by the APS, mosquitos are commonly found, mostly at warmer times of the year. Given that a WNV subtype has induced neurological disease

in Australian equine populations recently and that WNV can induce inflammatory CNS pathology similar to that observed with the APS, flaviviruses as a broad category should be considered in a refined differential diagnosis list for the APS.

4.5 Conclusion

Broiler chickens affected with the APS demonstrated sternal recumbency, stupor and marked flaccidity of the neck in the advanced stages of the syndrome. Histopathology associated with a proportion of affected chickens was characterised by non-suppurative vasculocentric encephalitis focussed in the cerebrum and Wallerian degeneration throughout the spinal cord. Based on the clinical presentation, associated pathology and other diagnostics performed, the differential diagnosis list has been narrow to include MD, AE and flaviviral disease (e.g. WNV) and thus diagnostics to test for the presence of the aetiological agents associated with each of these diseases should be incorporated into future investigations.

Chapter 5: Epidemiological investigations into an acute paralysis syndrome of broiler chickens

5.1 Introduction

This chapter provides an epidemiological description and analysis of an outbreak of an acute paralysis syndrome (APS) experienced over numerous broiler farms in an Australian production region. Chapter 4 provided a description of the clinical and pathological features of the APS and in doing so provided an indication as to a likely infectious aetiology to the APS, notably brain pathology consistent with other viral infections including Marek's disease (MD), avian encephalomyelitis (AE) and arboviral disease. However, as is the case with the majority of diseases whether or not infectious agents are implicated, aetiology is typically multifactorial and these factors need to be identified and understood in order for a disease to be effectively controlled and prevented.

Veterinary epidemiology is the science dealing with medicine in animal populations and more precisely the study of patterns of disease and health in animal populations (Toribio, 2008a). The aim of such is to provide insight into the distribution (spatial and temporal) and determinants (risk factors) of a disease, an understanding of which are essential in developing control and preventative strategies. Disease pathogenesis is often not straightforward which underlays the complexity of analytical techniques required for veterinary epidemiology (Lanzas & Chen, 2015). Logistic regression modelling is a commonly used technique for veterinary epidemiological analyses because it allows for the association between a set of risk factors (disease determinants) and a disease outcome (diseased or not diseased) to be quantified, adjusting for the presence of known confounders (Hosmer & Lemeshow, 2000). Univariate logistic regression analysis can provide a guide as to the association between one factor and an outcome; however, in many situations multiple factors are involved in the cause of disease, but this is not always the case (e.g. anthrax). Thus multivariate logistic regression modelling is considered most ideal in order to determine the relative importance of a range of possible factors in disease causation. The multivariate model is ideal in accounting for different distributions within levels of the outcome variable and association between independent variables (Hosmer & Lemeshow, 2000) and in doing so creating the most biologically reasonable model.

For this study, data were made available from farms in the affected region to allow for epidemiological analyses based on the recorded presence or absence of APS in each flock. This structure allowed for a retrospective case control study (Thrusfield, 2005) to be designed and performed. This study design was considered ideal given the availability of historical data, the need to quickly mount and conduct the study, the need to screen for multiple potential causative factors and the inability to manipulate factors at the farm level which prevented other farm-level study designs to be considered.

The approach to epidemiological modelling adopted for this study in order to identify and evaluate disease determinants for the APS was that of careful univariate analysis of individual factors in relation to the presence or absence of the APS before multivariate logistic regression analysis, adopted from Hosmer & Lemeshow (2000).

The objectives of this study were to:

- To describe the temporal and spatial distribution of the APS;
- To define the impact of the APS on the performance of broiler flocks;
- Identify factors and interactions between factors which were associated with the presence of the APS;
- Based on analyses, to devise mitigation strategies which could be quickly and relatively easily implemented at the farm level in order to assist with minimising the incidence of the APS in the absence of a definitive diagnosis; and
- To provide direction for further investigations into the causation of the APS.

5.2 Materials & Methods

5.2.1 Experimental design

A retrospective case-control study was conducted in which the broiler flock was the experimental unit. A flock was defined by a batch of chickens placed in a single shed on a farm at a point in time such that each shed could have had multiple flocks placed throughout the defined sampling period. For each flock the presence of the APS was defined by the observation of the clinical syndrome (Chapter 4) in one or more live broilers in a flock by senior farm managers or a veterinarian.

5.2.2 Dataset and overview of analytical techniques

Data were made available from farms in the production region in which the APS had been reported for flocks placed through the period 28th August 2012 and 5th December 2013. Data were

only used from flocks for which daily flock records were available and included farm and shed identification (ID), placement date (on the day of hatch), number of chickens placed, the presence or absence of the APS, shed size and design (new or old), flock sex status (as-hatched or sexed), daily mortality and cull counts, daily maximum and fluctuations in temperatures within the sheds, weekly average flock bodyweights (7, 14, 21, 28, 35, 42 and 49 days of age), age at thin-out times and parent flock and hatchery ID. The data set (Table 5.1) was transferred from a customised farm recording system to a spreadsheet (Microsoft Excel 2010). Daily maximum and fluctuations in temperature for the relevant region were accessed from the Australian Government Bureau of Meteorology (National Climate Centre, 2014) and incorporated into the dataset.

Flocks either had chickens placed as-hatched (AH) with males and females combined or sexed with males and females placed in separate pens within the shed. For sexed flocks a mean weekly flock bodyweight was calculated from the separate male and female bodyweight data to allow for comparison with AH flocks. The description of all sheds in the dataset is provided in Table 5.2. New sheds were defined by concrete flooring and the lining of internal walls, whereas old sheds had dirt floors and the internal walls were not lined. Flock sizes were defined as small (< 32,200 chickens), medium (32,200 – 44,400 chickens) and large (> 44,400 chickens) based on the division of the range in flock placement sizes into thirds. Analysis of data was performed using the statistical software JMP 11 (SAS Institute, 2013). Continuous variable data were assessed for normality and distribution of residuals to assess the need for transformation, from which none was required. A significance level of $P < 0.05$ was accepted for all test statistics.

Table 5.1 Summary of the dataset used for epidemiological analyses

Description	**Details**
Placement dates	28-Aug-2012 – 5-Dec-2013
Total number of chickens placed	13,828,088
-Total chickens as AH flocks	7,658,891
-Total chickens placed as sexed flocks	Males 3,013,580 Females 3,155,617
Farms included (number of flocks placed in sampling period)	1(80), 2(10), 3(30), 4(40), 5(20), 6(60), 9(20), 10(10), 11(20), 12(20), 13(48), 14(48)
Total flocks placed	406
Number of flocks with APS	29
Farms with APS (#flocks)	1(4), 9(1), 10(1), 13(7), 14(16)

Table 5.2 Description of sheds by farm in the dataset

Farm	Number of sheds	Floor space (m^2)	Design	Maximum placement size (chickens)
1	20	1,620 (10 sheds), 1,863 (10 sheds)	Old	30,120 (10 sheds), 34,704 (10 sheds)
2	10	1,620	Old	30,120
3	10	1,620	Old	30,120
4	10	1,620	Old	30,120
5	10	1,224	Old	22,716
6	10	1,500	Old	27,838
9	10	1,500	Old	27,838
10	10	1,500	Old	27,838
11	10	1,500	Old	27,838
12	10	1,500	Old	27,838
13	12	2,924	New	54,265
14	12	2,992	New	55,527

5.2.3 Describing the APS: temporal and spatial distribution

The temporal distribution of cases of the APS was graphed as three monthly rolling averages of the percentage of flocks placed which developed the APS by month. Spatial distribution of the APS was achieved by dividing each of the farms into clusters based on their relative location in relation to the local processing plant and feed mill. Contingency table analyses were performed on the proportions of flocks with and without the APS based on season of placement and cluster location. Logistic regression analysis was used to derive an OR in relation to the presence/absence of the APS in a flock for each season of placement. In order to do this reference cell coding was used with the season with the lowest incidence of the APS being set as the reference season.

5.2.4 Analysis of the effect of the APS on broiler flock performance

To assess the effect of the APS on broiler flock performance, continuous variable analyses of mortality, culls and bodyweight were performed using general linear models, fitting the effects of the presence/absence of the APS, flock sex status, shed design, flock size and season placed and interactions between these where considered appropriate. From these analyses, least-squares means (LSM) and standard errors (SEM) are presented and Tukey's HSD test was used to determine the significance of differences between means when there were more than two means. Mortality included birds which were found deceased whereas culls were birds requiring euthanasia and wastage was the derived combination of mortality and culls. The cause of each recorded mortality or cull was not defined in the dataset. Analysis of weekly bodyweight data were complicated by the sex status of flocks as bodyweight recordings in sexed flocks was separate for males and females; however, combined for AH flocks. Additionally, bodyweight data availability for females from sexed flocks was limited after 35 days of age due to thin-out practices. To allow for comparison between AH and sexed flocks, weekly bodyweights for sexed

flocks were calculated as an average between the male and female weekly weights through to 35 days of age.

5.2.5 Identification of disease determinants for the APS

In order to provide an estimate of the strength of association of potential risk factors to the APS, OR's were calculated. A positive association was determined when the OR was greater than 1 and this was regarded as significant if the 95% confidence interval exceeded unity. For dichotomous independent variables such as shed design, the OR described the risk of APS developing when the risk factor was present compared to when it was absent. For continuous independent variables such and temperatures and bodyweights, the OR described the risk of the APS developing with each unit increase or decrease (as indicated) in the independent variable. For uniformity in these analyses, units were derived for continuous independent variables which were one standard deviation in the dataset for that variable.

For continuous variables, general linear model analyses were performed in order to describe any variation present between flocks with and without the APS and for other factors as deemed relevant with respect to that variable. Additionally in relation to maximum and fluctuations in shed and regional temperatures, repeated measures analyses were performed using a mixed restricted maximum likelihood (REML) model, fitted for the effects of day, presence/absence of the APS, flock ID (fitted as a random effect term) and the interaction between day and presence/absence of the APS. Following these analyses univariate logistic regression analysis was performed in order to describe the risk (OR) of the APS developing with each unit increase or decrease in the independent variable.

For the categorical variables of season of placement, shed design, flock sex status, parent flocks and source hatcheries, chi-square analyses were performed to act as guide in determining the relevance of the proposed risk factor. If from such chi-square analyses there was deemed potential for confounding the Cochran-Mantel-Haenszel test was used to gain a result after data stratification with respect to the potential confounder. Subsequently, univariate logistic regression analysis was performed in order to describe the risk (OR) of the APS developing in the presence of the proposed risk factor compared to when it was absent.

5.2.6 Multivariate logistic regression analysis

In order to determine the relative importance of the risk factors derived from univariate analyses, a multivariate logistic regression model (LRM) was used. The approach to multivariate logistic regression was adopted from Hosmer & Lemeshow (2000) and comprised of the following steps:

1- Retained variables with a P-value < 0.25 from univariate logistic regression analyses described in 5.2.5 for step 2;
2- Ran a multivariate LRM and retained those variables which contributed to the model as determined by a Wald test-statistic < 0.25 and a relative increase in the estimated coefficient of each variable in the multivariate model compared to the coefficient from the univariate analysis for that variable;
3- Independently analysed each possible two-way interaction of the variables retained from step 2 in a multivariate LRM which included each of the retained independent variables and the two-way interaction being analysed. Interactions with likelihood ratio < 0.05 were retained for step 4;
4- Analysed each retained independent variable and interaction in a forward stepwise multivariate LRM and retained each variable and interaction with P < 0.05 for step 5; and
5- Performed a final logistic regression analysis in a LRM including the retained variables and interactions from step four.

The assumptions of logistic regression modelling were met by ensuring that all included variables were normally distributed (visual inspection), no important variables were omitted, no extraneous variables were included, independent variables could be measured without error and observations were independent and not linear combinations of one another. The Hosmer-Lemeshow goodness-of-fit test was used to assess model fit whereby a P-value closer to value 1 indicated best fit.

5.3 Results

5.3.1 Description of the APS: temporal and spatial distribution

5.3.1.1 Temporal distribution of the APS

The data presented in Figure 5.1 were derived from the calculation of three-monthly rolling averages for the percentages of flocks with the APS (left vertical axis) and total percentage flock mortality (right vertical axis) both by month and year of flock placement. The highest incidence of cases of the APS occurred in flocks placed in the months September – December and flock mortalities tended to be highest when greater proportions of flocks placed had the APS. Subsequent contingency analysis of the presence/absence of the APS by season of placement showed that there was a significant variation (P < 0.004) between seasons of placement in relation to the proportions of flocks with the APS: spring 20/159, summer 3/46, winter 4/93 and autumn 2/108.

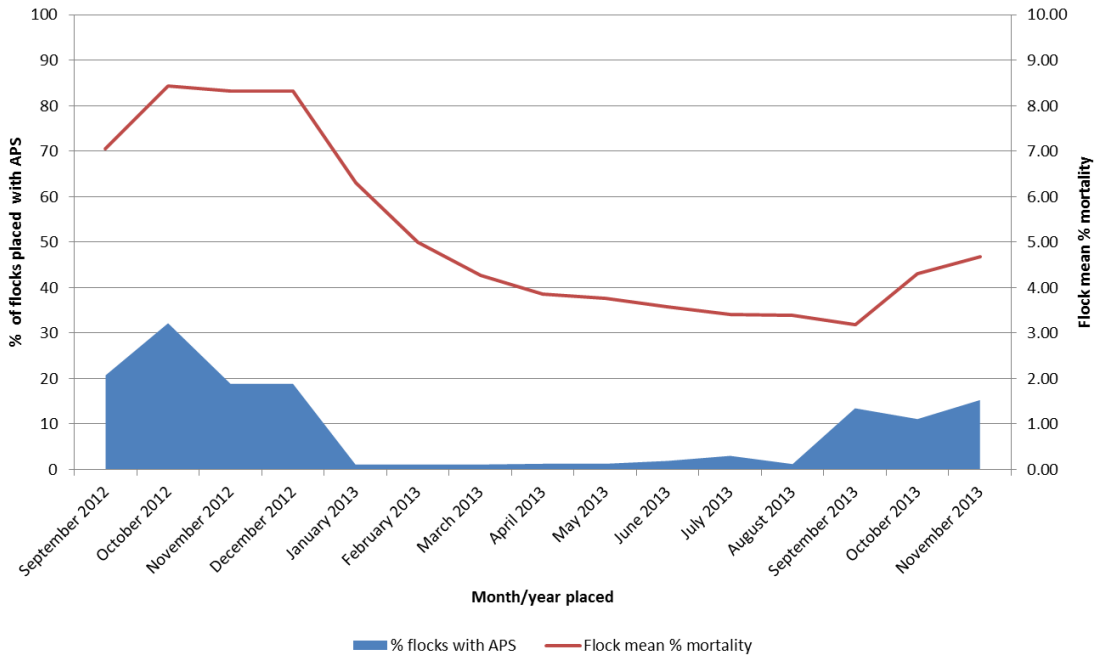

Figure 5.1 Three-monthly rolling averages for the percentage of flocks with the APS (blue-area) and mean percentage mortality of all flocks (red-line) by month/year of placement.

Logistic regression analysis was used to derive an OR in relation to the presence/absence of the APS in a flock for each season of placement. In order to do this reference cell coding was used with autumn being set as the reference month as it had the lowest incidence of the APS. Significant association was found between the presence of the APS and spring placement with spring placed flocks incurring 3.80 greater odds of developing the APS than flocks placed in other months (Summer OR 0.90, Winter OR 0.52) (Table 5.11).

5.3.1.2 Spatial distribution of the APS

The map presented in Figure 5.2 outlines the location of 14 of the broiler farms (Table 5.2) in proximity to the local feed mill and processing plant and divides the 14 farms into four clusters based on the direction of each cluster from the feed mill and processing plant. From the position of the feed mill and processing plant the approximate locations of each cluster are: cluster A (farms 2-6) south-south-east, cluster B (farms 1, 9-12) south-west, cluster C (farms 13-14) west and cluster D (farms 7-8) north-west. There was a significant effect ($P < 0.0001$) of cluster on the presence/absence of the APS which is evident in Figure 5.2 with farms in the data set with the APS (red-stars) only sitting in clusters B and C and with no affected farms sitting in cluster A. Unfortunately data from both farms in cluster D were not available in the data set, thus it is uncertain as to whether cases of the APS occurred in this cluster or not.

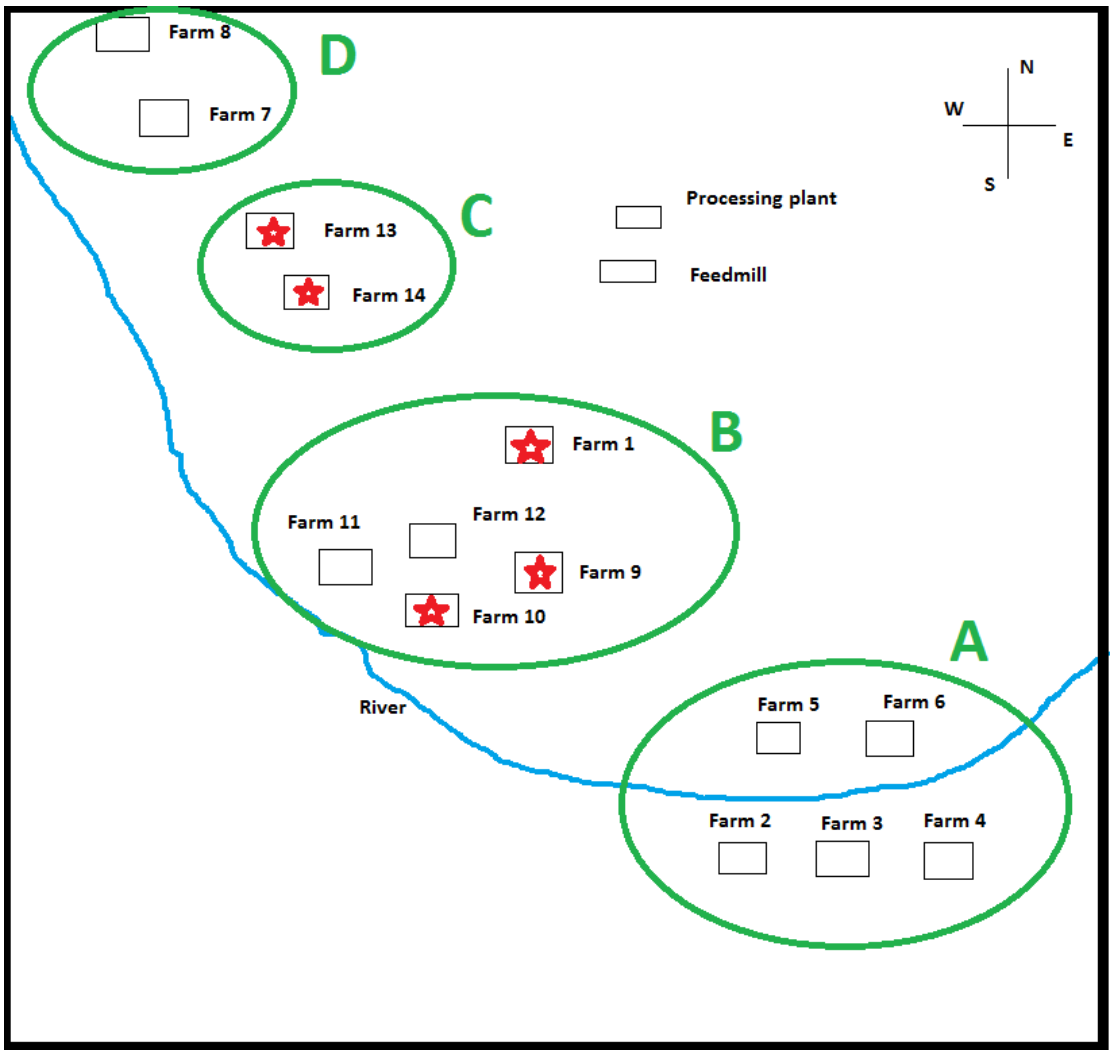

Figure 5.2 Division of 14 farms into clusters based on direction from the feed mill and processing plant, farms marked with a red star are those in the data set which reported the APS (not to scale).

Table 5.3 presents each flock in the dataset in which the APS had occurred and the times of placement and peak mortality for each alongside the mean regional average prevailing wind directions in the period between placement and peak mortality. This climatic information is based on averages through 1967 – 2010 (National Climate Centre, 2014). On visual analysis of the data presented in Table 5.3 there was no association between APS incidence and wind from the direction of the feed mill and processing plant.

Table 5.3 Monthly regional average prevailing wind directions (National Climate Centre, 2014) and their association with the period between placement and peak mortality in flocks with the APS

Farm	Shed	Placement date	Age at peak mortality (days)	Date at peak mortality	Prevailing 9 am wind: placement-peak mortality	Prevailing 3 pm wind: placement-peak mortality	Plant/mill direction from cluster
1	16	25-Jul-2013	34	28-Aug-2013	E	W/SW	NE
	18	01-Oct-2013	36	12-Nov-2013	SW	W/SW	NE
	16	03-Dec-2013	32	04-Jan-2014	SW	W/SW	NE
	18	03-Dec-2013	37	09-Jan-2014	SW	W/SW	NE
9	2	27-Aug-2013	35	01-Oct-2013	N	W	NE
10	7	07-Feb-2013	31	10-Mar-2013	E	SW	NE
13	3	30-Aug-2012	34	03-Oct-2012	N	W	E
	6	31-Aug-2012	39	09-Oct-2012	N	W	E
	8	05-Nov-2012	40	15-Dec-2012	SW	W/SW	E
	9	05-Nov-2012	44	19-Dec-2012	SW	W/SW	E
	12	06-Nov-2012	37	13-Dec-2012	SW	W/SW	E
	10	24-May-2013	39	02-Jul-2013	E	SW	E
	12	27-May-2013	29	25-Jun-2013	E	SW	E
14	3	04-Sep-2012	36	10-Oct-2012	N/SW	W	E
	4	04-Sep-2012	42	16-Oct-2012	N/SW	W	E
	5	05-Sep-2012	41	16-Oct-2012	N/SW	W	E
	8	06-Sep-2012	33	09-Oct-2012	N/SW	W	E
	7	06-Sep-2012	39	15-Oct-2012	N/SW	W	E
	9	07-Sep-2012	41	18-Oct-2012	N/SW	W	E
	1	07-Nov-2012	41	18-Dec-2012	SW	W/SW	E
	2	08-Nov-2012	39	17-Dec-2012	SW	W/SW	E
	3	08-Nov-2012	40	18-Dec-2012	SW	W/SW	E
	7	09-Nov-2012	42	21-Dec-2012	SW	W/SW	E
	4	09-Nov-2012	38	17-Dec-2012	SW	W/SW	E
	6	09-Nov-2012	38	17-Dec-2012	SW	W/SW	E
	8	12-Nov-2012	39	21-Dec-2012	SW	W/SW	E
	9	12-Nov-2012	41	23-Dec-2012	SW	W/SW	E
	10	13-Nov-2012	38	21-Dec-2012	SW	W/SW	E
	11	13-Nov-2012	40	23-Dec-2012	SW	W/SW	E

5.3.2 Analysis of the effect of the APS on broiler flock performance

5.3.2.1 Mortality, culls and wastage

Continuous variable analyses of flock factors in relation to mortality, culls and total wastage (mortality and culls) are provided in Table 5.4. Total flock mortality, cull and wastage rates (as a percentage of chickens placed) were significantly greater in flocks with the APS compared to flocks without the APS (Table 5.4).

Figure 5.3 plots the mean daily mortality for flocks by presence/absence of the APS showing peaks in mortality at 28, 37, 39 and 46 days of age in flocks with the APS. Similar peaks in

mortality were not evident in flocks without the APS. The majority of mortalities in flocks with the APS occurred between 26 - 51 days of age.

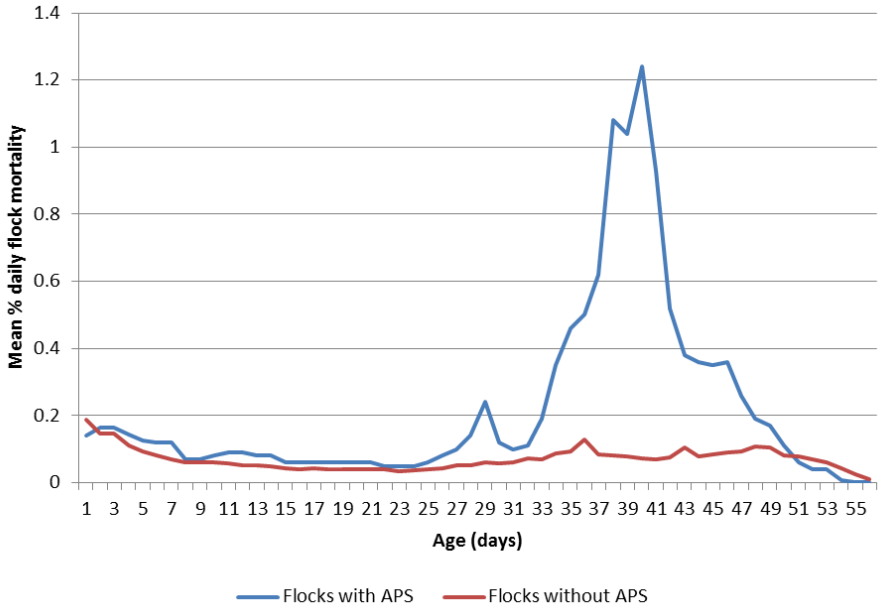

Figure 5.3 Mean percentage daily mortality of flocks with (blue line) and without (red line) the APS

Sexed flocks suffered significantly higher mortalities than AH flocks and the interaction between sex status and presence/absence of the APS was significant with sexed flocks with the APS suffering the highest mortality rate (Table 5.4). Within sexed flocks male but not female mortality was increased significantly in flocks with the APS ($P < 0.0001$) (Figure 5.4).

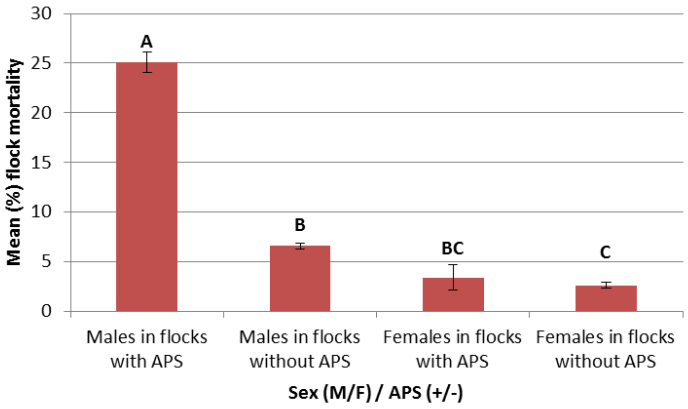

Figure 5.4 LSM ± SEM flock mortality (%) showing effect of the interaction between sex and the presence/absence of the APS within sexed flocks, columns not sharing a common letter differ significantly

Flocks in new sheds suffered significantly higher mortalities than those in old sheds and the interaction between shed design and the presence/absence of the APS was significant with flocks in new sheds with the APS suffering the highest mortality rate (Table 5.4). Significantly higher mortality rates were recorded for large flocks than for small and medium sized flocks and the interaction between flock size and the presence/absence of the APS was significant with large

flocks with the APS having the highest mortality rates (Table 5.4). Season of placement had a significant effect on mortality rates with the highest mortality rates recorded for flocks placed in spring, which is demonstrated in Figure 5.1. The effect on mortality from the interaction between season of placement and the presence/absence of the APS was significant with flocks with the APS placed in autumn, winter and spring having the highest mortality rates (Table 5.4). The effect on mortality from the interaction between season of placement and flock sex status was also significant, with sexed flocks placed in spring having the highest mortality rates (Table 5.4).

Figure 5.4 plots the mean daily cull rates for flocks with and without the APS and demonstrates peaks in cull rates at 37, 40, 44, 47 and 50 days of age in flocks with the APS. The curve also demonstrates that the majority of culls in flocks with the APS occurred in the period 26-51 days of age which corresponded with the majority of mortalities in these flocks as shown in Figure 5.3.

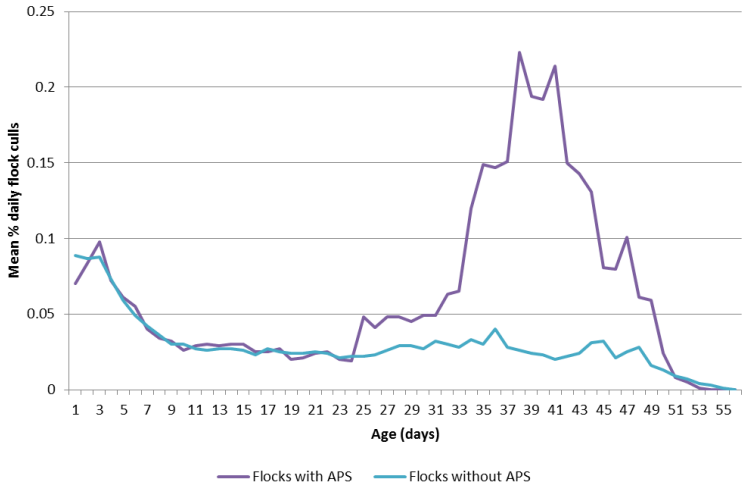

Figure 5.5 Mean daily percentage culls of flocks with (purple line) and without (blue line) the APS

AH flocks suffered significantly higher cull rates than sexed flocks (Table 5.4). Season of placement had a significant effect on cull rates with flocks placed in spring having the highest cull rates (Table 5.4). The effect on cull rates from the interaction between season of placement and the presence/absence of the APS was significant with flocks with the APS placed in spring having the highest cull rates (Table 5.4). The interaction between sex status and shed design had a significant effect on flock cull rates with sexed flocks in new sheds having the highest cull rates (Table 5.4). The interaction between sex status and flock size had a significant effect on flock cull rates with medium sized AH flocks having the highest cull rates (Table 5.4). The effect on cull rates from the interaction between season of placement and flock sex status was also significant, with AH flocks placed in summer having the highest cull rates (Table 5.4).

Table 5.4 Continuous variable analyses of flock factors in relation to mortality, culls, wastage and weekly bodyweights

Variable (effect and level)	Number of flocks (n)	Mortality (%)	Culls (%)	Wastage (%)	7 day weight (g)	14 day weight (g)	21 day weight (g)	28 day weight (g)	35 day weight (g)
Presence/absence of APS (P-value)		**<0.0001**	**<0.0001**	**<0.0001**	0.43	**<0.0001**	**<0.0001**	**0.0009**	0.09
APS present	29	12.3±0.5	3.60±0.20	15.9±0.6	181.72±2.55	489.1±6.3	934±12	1484±16	2007±20
APS absent	377	3.9±0.1	1.64±0.05	5.5±0.2	179.63±0.71	462.7±1.7	882±3	1427±5	1972±5.4
Sex status of flocks (P-value)		**<0.0001**	**0.03**	**0.0002**	**0.04**	**0.002**	**0.0005**	**<0.0001**	**<0.0001**
AH	243	3.78±0.22	1.623±0.091	7.23±0.32	178.6±0.9	460.2±2.2	876.6±4.2	1414±6	1942±6
Sexed	163	5.60±0.26	1.882±0.075	5.66±0.27	181.5±1.1	471.1±2.7	900.0±5.1	1457±7	2023±8
Shed design (P-value)		**<0.0001**	0.34	**<0.0001**	**0.002**	**<0.0001**	**<0.0001**	**0.0004**	**0.0003**
New	96	6.46±0.34	1.877±0.119	8.33±0.41	183.5±1.4	482.0±3.4	916±7	1459±9	2008±11
Old	310	3.91±0.19	1.747±0.066	5.66±0.23	178.6±0.8	459.1±1.9	877±4	1423±5	1964±6
Flock size (P-value)		**<0.0001**	0.55	**<0.0001**	**0.004**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Small	277	3.96 ± 0.20^{B}	1.735±0.070	5.69 ± 0.24^{B}	179.0 ± 0.8^{B}	462.3 ± 1.9^{B}	886 ± 4^{B}	1429 ± 5^{B}	1969 ± 6^{B}
Medium	33	3.53 ± 0.58^{B}	1.852±0.204	5.38 ± 0.71^{B}	175.4 ± 2.4^{B}	432.7 ± 5.6^{C}	802 ± 10^{C}	1368 ± 15^{C}	1922 ± 18^{C}
Large	96	6.46 ± 0.34^{A}	1.877±0.120	8.33 ± 0.41^{A}	183.5 ± 1.4^{A}	482.0 ± 3.3^{A}	916 ± 6^{A}	1459 ± 9^{A}	2008 ± 11^{A}
Season placed (P-value)		**0.0009**	**0.01**	**0.0007**	0.74	0.11	**0.0001**	**<0.0001**	**0.0001**
Autumn	108	3.79 ± 0.33^{B}	$1.49\pm0.11\ \%^{B}$	5.29 ± 0.40^{B}	181.01±1.32	460.7±3.3	869 ± 6^{B}	1424 ± 8^{BC}	2000 ± 9^{B}
Winter	93	3.87 ± 0.36^{B}	$1.83\pm0.12\ \%^{AB}$	5.70 ± 0.43^{B}	178.98±1.43	463.0±3.6	873 ± 7^{B}	1397 ± 9^{C}	1901 ± 10^{C}
Spring	159	5.31 ± 0.27^{A}	$1.97\pm0.09\ \%^{A}$	7.29 ± 0.33^{A}	179.54±1.10	469.6±2.7	899 ± 5^{A}	1446 ± 7^{AB}	1978 ± 8^{B}
Summer	46	4.72 ± 0.51^{AB}	$1.68\pm0.17\ \%^{AB}$	6.41 ± 0.61^{AB}	179.33±2.03	459.4±5.1	904 ± 10^{A}	1464 ± 13^{A}	2046 ± 14^{A}
Presence/absence of the APS * Sex status (P-value)		**0.002**	0.12	**0.002**	0.42	0.16	0.21	0.96	0.19
APS present and sexed	17	14.1 ± 0.6^{A}	3.69 ± 0.25^{A}	17.8 ± 0.8^{A}	184.5±3.3	500 ± 8^{A}	955 ± 16^{A}	1500 ± 21^{A}	2064 ± 25^{A}
APS present and AH	12	9.7 ± 0.8^{B}	3.48 ± 0.30^{A}	13.2 ± 0.9^{B}	177.8±4.0	474 ± 10^{AB}	$905\pm19^{AB C}$	1461 ± 25^{AB}	1936 ± 28^{B}
APS absent and sexed	146	4.6 ± 0.2^{C}	1.38 ± 0.09^{C}	6.0 ± 0.3^{C}	181.1±1.1	468 ± 3^{B}	894 ± 5^{B}	1452 ± 7^{A}	2019 ± 8^{A}
APS absent and AH	231	3.4 ± 0.2^{D}	1.80 ± 0.07^{B}	5.3 ± 0.2^{C}	178.7±0.9	460 ± 2^{B}	875 ± 4^{C}	1411 ± 6^{B}	1942 ± 6^{B}
Presence/absence of the APS * Shed design (P-value)		**<0.0001**	0.38	**<0.0001**	0.76	0.29	0.54	0.64	0.49
APS present and new shed	23	13.7 ± 0.6^{A}	3.61 ± 0.22^{A}	17.3 ± 0.7^{A}	182.4 ± 2.8^{AB}	496 ± 7^{A}	944 ± 13^{A}	1493 ± 18^{A}	2009 ± 23^{AB}
APS present and old shed	6	6.8 ± 1.1^{B}	3.55 ± 0.43^{A}	10.4 ± 1.3^{B}	179.2 ± 5.6^{AB}	461 ± 13^{AB}	895 ± 26^{AB}	1448 ± 36^{AB}	1999 ± 42^{AB}
APS absent and new shed	73	4.2 ± 0.3^{BC}	1.33 ± 0.12^{C}	5.5 ± 0.4^{C}	183.8 ± 2^{A}	478 ± 4^{A}	907 ± 7^{A}	1448 ± 10^{AB}	2008 ± 12^{A}
APS absent and old shed	304	3.9 ± 0.2^{C}	1.71 ± 0.06^{B}	5.6 ± 0.2^{C}	178.6 ± 0.8^{B}	459 ± 2^{B}	876 ± 4^{B}	1422 ± 5^{B}	1963 ± 6^{B}

Variable (effect and level)	**Number of flocks (n)**	**Mortality (%)**	**Culls (%)**	**Wastage (%)**	**7 day weight (g)**	**14 day weight (g)**	**21 day weight (g)**	**28 day weight (g)**	**35 day weight (g)**
Presence/absence of the APS * Flock size (P-value)		**<0.0001**	0.42	**<0.0001**	0.94	0.50	0.89	0.68	0.20
APS present and Large	23	13.7±0.6^{A}	3.6±0.2^{A}	17.3±0.7^{A}	182.4±2.8^{AB}	496±7^{A}	944±12^{A}	1493±18^{A}	2009±22^{AB}
APS present and Medium	1	5.3±2.7^{B}	4.6±1.1^{AB}	9.8±3.2^{ABC}	175.0±13.6^{AB}	455±32^{ABC}	822±60$^{AB C}$	1336±86^{ABC}	1810±10^{ABC}
APS present and Small	5	7.1±1.2^{B}	3.4±0.5^{A}	10.5±1.4^{B}	180.0±6.1^{AB}	462±14^{ABC}	909±27^{AB}	1470±38^{ABC}	2037±46^{ABC}
APS absent and Large	73	4.2±0.3^{B}	1.3±0.1^{C}	5.5±0.4^{C}	183.8±1.6^{A}	478±4^{A}	907±7^{AB}	1448±10^{AB}	2008±12^{A}
APS absent and Medium	32	3.5±0.5^{B}	1.8±0.2^{BC}	5.2±0.6^{C}	175.4±2.4^{B}	432±6^{C}	801±11^{C}	1369±15^{C}	1925±18^{C}
APS absent and Small	272	3.9±0.2^{B}	1.7±0.1^{BC}	5.6±0.2^{C}	179.0±0.8^{AB}	462±2^{B}	885±4^{B}	1428±5^{B}	1968±6^{BC}
Presence/absence of the APS * Season placed (P-value)		**0.0008**	**0.008**	**0.002**	0.99	0.81	0.34	**0.01**	0.62
APS present and Autumn	2	14.6±1.9^{A}	1.90±0.74 %^{AB}	16.5±2.3^{AB}	182.0±9.8	473±24^{AB}	848±45^{AB}	1302±60^{CE}	1966±95^{ABC}
APS present and Winter	4	12.7±1.3^{A}	2.89±0.52 %^{AB}	15.5±1.6^{AB}	182.0±6.9	488±17^{AB}	900±32^{AB}	1414±42$^{ABCD E}$	1913±47^{ABC}
APS present and Spring	20	12.8±0.6^{A}	4.06±0.23 %^{A}	16.9±0.7^{A}	181.3±3.1	494±8^{A}	950±14^{A}	1509±19^{A}	2009±22^{AB}
APS present and Summer	3	6.7±1.6^{B}	2.63±0.60 %^{AB}	9.3±1.9^{BC}	184.0±8.0	469±20^{AB}	930±37^{AB}	1540±49^{ABD}	2132±55^{AB}
APS absent and Autumn	106	3.6±0.3^{CD}	1.49±0.10 %^{B}	5.1±0.3^{C}	181.0±1.3	460±3^{B}	870±6^{B}	1427±8^{BCDE}	2001±9^{AB}
APS absent and Winter	89	3.5±0.3^{D}	1.78±0.11 %^{B}	5.3±0.3^{C}	178.8±1.5	462±4^{B}	872±7^{B}	1397±9DE	1900±10^{C}
APS absent and Spring	139	4.2±0.2^{BC}	1.67±0.09 %^{B}	5.9±0.3^{C}	179.3±1.2	466±3^{B}	892±5^{B}	1437±7^{BC}	1974±8^{B}
APS absent and Summer	43	4.6±0.4^{B}	1.62±0.16 %^{B}	6.2±0.5^{C}	179.0±2.1	459±5^{B}	902±10^{AB}	1459±13^{ABC}	2040±14^{A}
Sex status * Shed design (P-value)		0.73	**0.007**	0.29	0.65	0.66	0.63	**0.01**	0.27
Sexed and New	62	7.0±0.4^{A}	1.97±0.15 %^{A}	9.0±0.5^{A}	184.5±1.7^{A}	483.1±4.2^{A}	919±8^{A}	1458±11^{A}	2028±12^{A}
Sexed and Old	101	4.7±0.3^{B}	1.41±0.11 %^{B}	6.2±0.4^{BC}	179.6±1.4^{B}	463.6±3.3^{BC}	888±6^{BC}	1457±9^{A}	2020±10^{AB}
AH and New	34	5.5±0.6^{B}	1.70±0.20 %^{AB}	7.2±0.7^{B}	181.6±2.3^{AB}	480.1±5.7^{AB}	910±11^{AB}	1461±15^{A}	1972±17^{BC}
AH and Old	209	3.5±0.2^{C}	1.91±0.08 %^{A}	5.4±0.3^{C}	178.2±0.9^{B}	457.0±2.3^{C}	871±4^{C}	1406±6^{B}	1937±7^{C}
Sex status * Flock size (P-value)		0.43	**0.03**	0.32	0.32	0.22	0.89	**0.03**	0.47
Sexed and Small	92	4.9±0.3^{B}	1.41±0.12 %^{B}	6.3±0.4^{B}	179.4±1.4^{AB}	465±3^{BC}	895±6^{AB}	1460±9^{A}	2022±10^{A}
Sexed and Medium	9	3.3±1.1^{BC}	1.42±0.39 %^{AB}	4.7±1.3^{B}	181.7±4.5^{AB}	452±11^{ABCD}	818±20^{C}	1422±28^{AB}	1997±32^{ABC}
Sexed and Large	62	7.0±0.4^{A}	1.97±0.15 %^{A}	9.0±0.5^{A}	184.5±1.7^{A}	483±4^{A}	919±8^{A}	1458±11^{A}	2028±12^{A}
AH and Small	185	3.5±0.2^{C}	1.90±0.08 %^{A}	5.4±0.3^{B}	178.8±1.0^{B}	461±2^{C}	881±4^{B}	1414±6^{B}	1943±7^{BC}
AH and Medium	24	3.6±0.7^{BC}	2.01±0.24 %^{A}	5.6±0.8^{B}	173.0±2.8^{B}	426±7^{D}	796±12^{C}	1348±17^{C}	1893±20^{C}
AH and Large	34	5.5±0.6^{AB}	1.70±0.20 %^{AB}	7.2±0.7^{AB}	181.6±2.3^{AB}	480±5^{AB}	910±10^{AB}	1461±14^{A}	1972±16^{AB}

Variable (effect and level)	**Number of flocks (n)**	**Mortality (%)**	**Culls (%)**	**Wastage (%)**	**7 day weight (g)**	**14 day weight (g)**	**21 day weight (g)**	**28 day weight (g)**	**35 day weight (g)**
Sex status * Season placed (P-value)		**0.0001**	**0.002**	**0.0009**	0.20	**<0.0001**	0.07	**0.007**	**0.005**
AH and Autumn	39	3.4±0.5^{B}	1.34±0.18 %^{BC}	4.7±0.6^{B}	177.5±2.2	455±5^{C}	860±10^{C}	1419±13^{CD}	1979±14^{BC}
AH and Winter	87	3.7±0.4^{B}	1.90±0.12 %^{ABC}	5.6±0.4^{B}	178.3±1.5	458±4^{C}	868±7^{BC}	1389±9^{D}	1889±10^{D}
AH and Spring	96	3.8±0.3^{B}	2.00±0.12 %^{AB}	5.8±0.4^{B}	179.7±1.4	466±3^{BC}	885±6^{BC}	1431±9^{C}	1959±9^{C}
AH and Summer	21	4.8±0.7^{B}	2.29±0.25 %^{A}	7.1±0.9^{AB}	177.0±3.0	454±7^{BC}	902±14^{ABC}	1430±18^{BC}	2014±20^{ABC}
Sexed and Autumn	69	4.0±0.4^{B}	1.58±0.14 %^{ABC}	5.6±0.9^{B}	183.0±1.6	464±4^{BC}	874±8^{BC}	1427±10^{C}	2013±11^{AB}
Sexed and Winter	6	6.7±1.3^{AB}	0.79±0.46 %^{ABC}	7.5±1.6^{AB}	188.3±5.6	538±13^{A}	949±26^{AB}	1526±34^{A}	2075±37^{ABC}
Sexed and Spring	63	7.6±0.4^{A}	1.93±0.14 %^{ABC}	9.5±0.5^{A}	179.3±1.7	475±4^{B}	921±8^{A}	1470±11^{AB}	2009±12^{AB}
Sexed and Summer	25	4.6±0.6^{B}	1.17±0.23 %^{C}	5.8±0.8^{B}	181.2±2.7	464±7^{BC}	906±13^{ABC}	1494±17^{A}	2073±18^{A}
Shed design * Flock size (P-value)		n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}
Shed design * Season placed (P-value)		n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}
Flock size * Season placed (P-value)		n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}	n/a^{1}

n/a^{1}Insufficient degrees of freedom to perform analysis

5.3.2.2 Bodyweight

Bodyweights of flocks with the APS were significantly greater than for flocks without the APS at 14, 21 and 28 days of age (Figure 5.6, Table 5.4). At each of these three ages the mean weight of flocks with the APS exceeded the Ross 308 target weights (Aviagen, 2012) and flocks without the APS were below the Ross 308 target weights (Figure 5.6). At seven and 35 days of age all flocks were below the Ross 308 target weights (Figure 5.6).

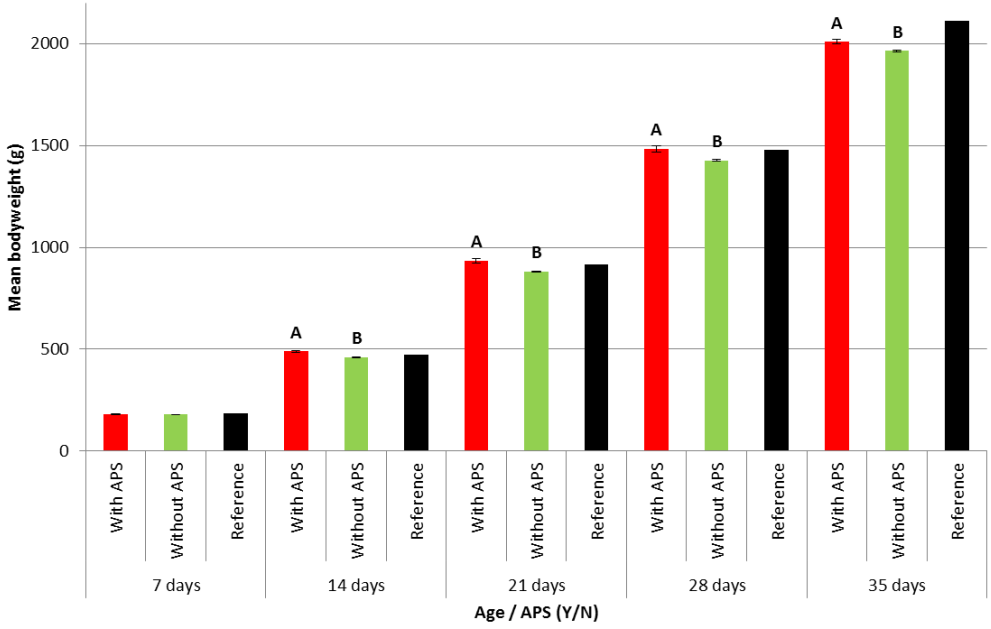

Figure 5.6 Mean weekly flock bodyweights by presence/absence of the APS (target AH Ross 308 weights are black columns). Columns with a different letter differ significantly (applicable only for each age).

Bodyweights of flocks reared in new sheds were significantly heavier than those in old sheds at all ages (Table 5.4). Similarly large flocks were significantly heavier than medium and small flocks at all ages (Table 5.4) which corresponded with shed design as all large flocks were reared in new sheds. Bodyweights of sexed flocks were significantly heavier than AH flocks at all ages (Table 5.4). Bodyweights of flocks placed in spring and summer were heavier than flocks placed in winter at 21, 28 and 35 days of age (Table 5.4). At 28 days of age the interaction between the presence/absence of the APS and season placed was significant, with flocks with the APS placed in autumn recording the lowest weights (Table 5.4). Additionally at this age the effect on bodyweight from the interactions between shed design and flock sex status and flock size and flock sex status were significant with AH flocks reared in old sheds and small and medium AH flocks recording the lowest weights respectively (Table 5.4). The interaction between flock sex status and season of placement on bodyweight was significant at 14, 28 and 35 days of age with sexed flocks placed in winter being heaviest (Table 5.4).

5.3.3 Identification of disease determinants for the APS

5.3.3.1 High growth rates

Table 5.5 outlines the OR associated with unit increases in mean weekly flock bodyweight above the Ross 308 standard performance guidelines and the presence of the APS across all flocks and also for males and females specifically within sexed flocks. It is evident that the age at which the highest risk of the APS developing from increases in mean flock bodyweight above the standard performance guidelines was 14 days of age, at which point each unit increase in bodyweight above the guidelines was associated with 2.26, 3.88 and 2.09 times increased odds of the APS developing across all flocks and in males and females (from sexed flocks) respectively.

Table 5.5 Odds ratios associated with weekly bodyweight increases above the Ross 308 standard performance guidelines (Aviagen, 2012) and the presence of the APS

Age (days)	Ross 308 standard performance guidelines	Mean (one standard deviation) bodyweight	OR (95% CI) of APS per standard deviation increase above guidelines	P-value
All flocks (AH and sexed flocks included)				
7	185 g	180 (14) g	1.17 (0.80, 1.77)	0.42
14	473 g	465 (34) g	**2.26** (1.52, 3.46)	**< 0.0001**
21	916 g	886 (66) g	**2.20** (1.50, 3.29)	**< 0.0001**
28	1,479 g	1,431 (89) g	**1.86** (1.28, 2.76)	**0.001**
35	2,113 g	1,974 (105) g	1.40 (0.95, 2.07)	0.09
Males within sexed flocks				
7	186 g	185 (13) g	1.24 (0.75, 2.14)	0.40
14	481 g	486 (37) g	**3.88** (2.12, 7.84)	**<0.0001**
21	945 g	942 (78) g	**3.04** (1.80, 5.55)	**<0.0001**
28	1,553 g	1,548 (101) g	**2.27** (1.31, 4.16)	**0.003**
35	2,250 g	2,163 (128) g	**2.47** (1.40, 5.17)	**0.001**
42	2,979 g	2,833 (187) g	**3.32** (1.77, 6.86)	**<0.0001**
49	3,695 g	3,381 (144) g	**2.33** (1.25, 4.45)	**0.008**
Females within sexed flocks				
7	185 g	177 (13) g	1.38 (0.83, 2.37)	0.22
14	466 g	455 (36) g	**2.09** (1.18, 3.89)	**0.01**
21	886 g	857 (62) g	**1.80** (1.07, 3.15)	**0.03**
28	1,406 g	1,367 (75) g	1.30 (0.78, 2.27)	0.32
35	1,977 g	1,884 (86) g	0.73 (0.42, 1.25)	0.26

OR: odds ratio, CI: confidence interval, APS: acute paralysis syndrome, AH: as-hatched.

5.3.3.2 Shed design

The breakdown of the dataset with respect to shed design on each farm was that all sheds in the data set from farms 13 and 14 were of new design and all sheds from the other farms were of old design (Table 5.2). The proportion of flocks placed in new sheds with the APS (23/96) was found to be significantly greater ($P < 0.0001$) than that for flocks placed in old sheds with the APS (6/310). Flocks placed in new sheds had 15.96 greater odds (Table 5.11) of developing the APS than did flocks placed in old shed. This result could have been confounded by farm given that all

flocks placed in new sheds were on farms 13 and 14; however, as there were not any farms with both new and old sheds a CMH test could not be performed to measure this.

5.3.3.3 Flock sex status

Contingency analysis of the presence/absence of the APS by flock sex status showed that the APS was observed in a significantly greater (P = 0.04) proportion of sexed flocks (17/163) compared to AH flocks (12/243). Sexed flocks experienced 2.24 (1.05, 4.94) greater odds (Table 5.11) of developing the APS than did AH flocks. However, relatively high proportions of the flocks placed on farms 13 (75%) and 14 (54%) were sexed flocks. Given that farms 13 and 14 accounted for the majority of cases of the APS (Table 5.1), farm ID could have confounded this result. As a result the CMH test was performed with data stratified by farm, confirming this confounding (P = 0.68).

5.3.3.4 Flock size

Contingency analysis of the presence/absence of the APS by flock size showed that there was a significant variation (P < 0.0001) between flock sizes in relation to the proportions of flocks with the APS: large flocks 23/96, medium flocks 1/33 and small flocks 5/277. A level of confounding was confirmed with respect to this result from the CMH test (P = 0.50) because all large flocks were isolated to farms 13 and 14, which accounted for the majority of APS cases. Univariate logistic analysis found that for each SDU increase (10,827 chickens) in flock size the odds of the APS developing in a flock increased 3.44 times (Table 5.11).

5.3.3.5 Shed temperature

Analyses relating to daily maximum and fluctuations in shed temperature are summarised in Table 5.6. Two periods were selected for initial analysis of shed temperatures including placement - 25 days of age which was the period leading into the first observed spike in mortality in flocks with APS (Figure 5.3) and 26-51 days of age which was the period in which the majority of mortalities occurred in flocks with the APS (Figure 5.3).

Figure 5.7 plots the daily maximum shed temperature by age for flocks with and without the APS. Repeated measures analysis showed significant effects of both day (P < 0.0001) and the presence/absence of the APS (P < 0.0001), highlighting that maximum shed temperatures varied significantly throughout the age of flocks and also between flocks by presence/absence of the APS. Additionally, the effect on mean maximum shed temperatures from the interaction between day and the presence or absence of the APS was also significant (P < 0.0001), thus confirming that variations in daily maximum shed temperatures between flocks with and without the APS were age dependant.

The difference in mean daily maximum shed temperature between flocks with and without the APS was non-significant in the period placement - 25 days of age; however, significant in the period 26-51 days in which it was higher in flocks with the APS (Table 5.6). Older sheds experienced significantly higher mean maximum daily shed temperatures than did new sheds in the first 25 days of age; however, the temperatures were similar between new and old shed through the period 26-51 days of age (Table 5.6). This corresponded with the effect of flock size on mean daily maximum shed temperature whereby the temperature associated with small and medium sized flocks (relatively smaller sheds) were significantly greater than for large flocks (larger sheds) (Table 5.6) in the period placement – 25 days of age but not 26-51 days of age. Mean daily maximum shed temperatures were highest in flocks placed in spring and summer (Table 5.6).

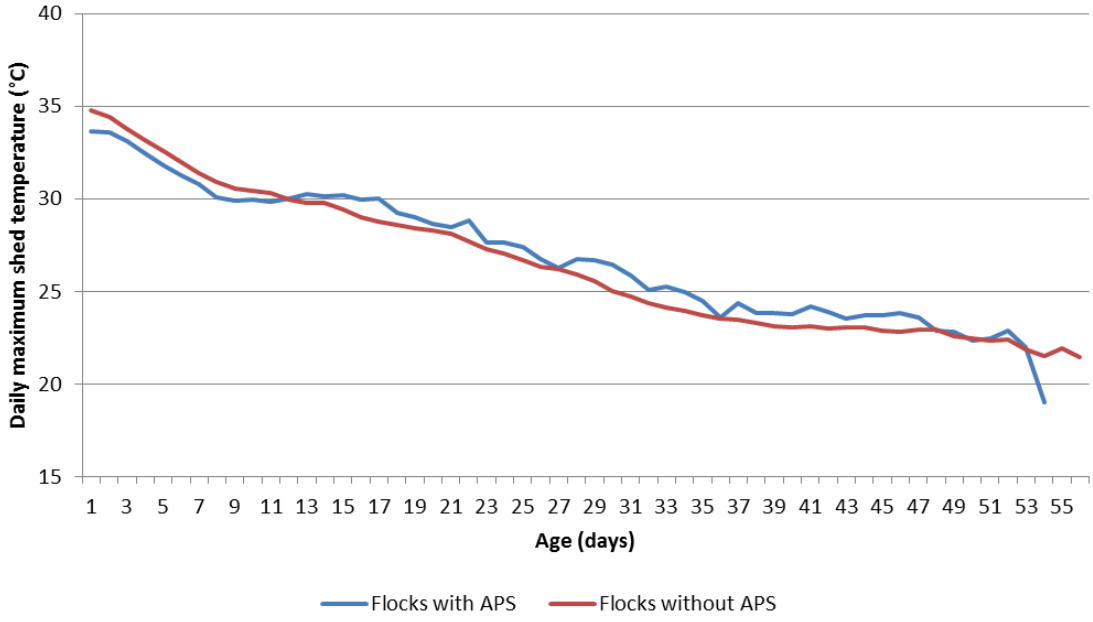

Figure 5.7 Mean daily maximum shed temperatures associated with flocks with (blue line) and without (red line) the APS

Figure 5.8 plots the mean daily shed temperature fluctuations by age for flocks with and without the APS. Repeated measures analysis showed significant effects of both day ($P < 0.0001$) and the presence/absence of the APS ($P < 0.0001$) highlighting that mean shed temperature fluctuations varied significantly throughout the age of the flock and also between flocks with and without the APS. Additionally the effect on mean shed temperature fluctuations from the interaction between day and the presence or absence of the APS was also significant ($P < 0.0001$) thus confirming that variations in daily shed temperature fluctuations between flocks with and without the APS were age dependant.

Mean daily fluctuations in shed temperatures were significantly greater in flocks with the APS than flocks without the APS through both periods placement - 25 and 26-51 days of age (Table 5.6). Additionally, through both periods, shed temperature fluctuations were greatest in new sheds, large flocks and flocks placed in spring and summer (Table 5.6). The effect on mean daily shed temperature fluctuation from the interaction between the presence/absence of the APS and season placed was significant in both periods (Table 5.6). This was due to significant variation in temperature fluctuations in spring placed flocks with the APS being greater than spring placed flocks without the APS in the period placement – 25 days of age and due to temperature fluctuations in autumn and winter flocks with the APS being greater than autumn and winter placed flocks without the APS through 26-51 days of age (Table 5.6).

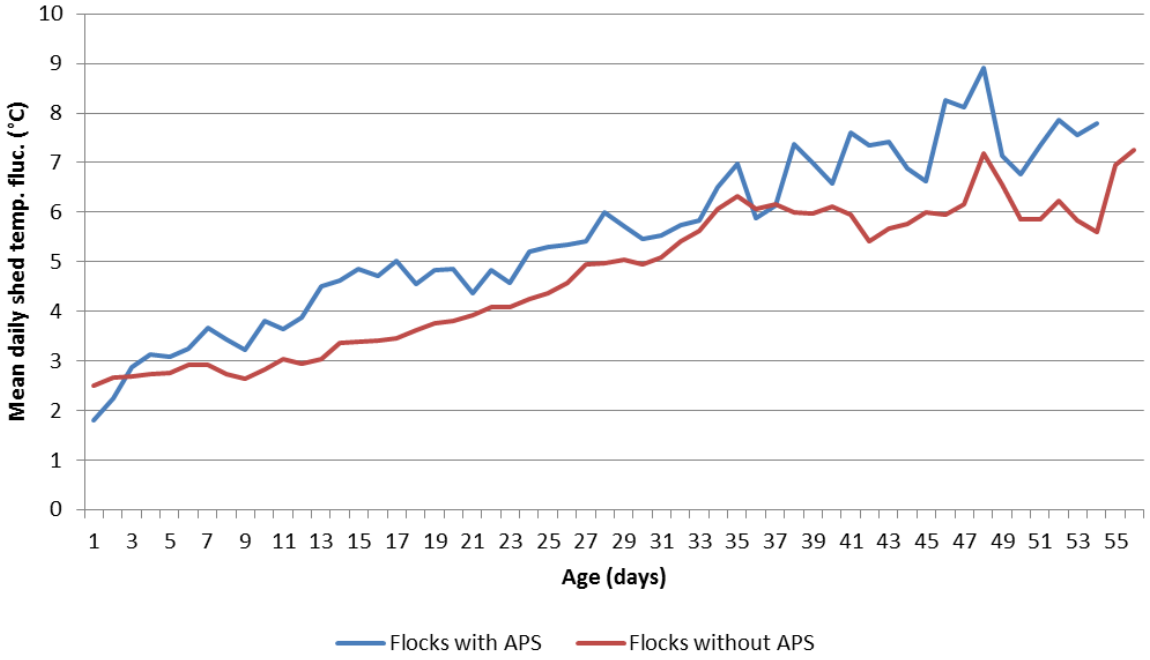

Figure 5.8 Mean daily fluctuations in shed temperatures associated with flocks with (blue line) and without (red line) the APS

Table 5.6 Continuous variable analyses of flock factors in relation to maximum and fluctuations in shed temperatures

Variable (effect and level)	**Number of flocks (n)**	**Mean maximum shed temp. 0-25 days of age**	**Mean maximum shed temp. 26-51 days of age**	**Mean fluctuation in shed temp. 0-25 days of age**	**Mean fluctuation in shed temp. 26-51 days of age**
Presence/absence of APS (P-value)		0.09	**0.008**	**<0.0001**	**0.004**
APS present	29	30.150±0.221°C	24.80±0.35°C	4.01±0.15°C	6.48±0.24°C
APS absent	377	30.116±0.062°C	23.82±0.10°C	3.28±0.04°C	5.77±0.07°C
Shed design (P-value)		**<0.0001**	0.27	**0.04**	**0.0001**
New	96	29.67±0.12°C	23.707±0.192°C	3.479±0.083°C	6.25±0.13°C^{A}
Old	310	30.27±0.07°C	23.950±0.110°C	3.283±0.048°C	5.68±0.07°C^{B}
Flock size (P-value)		**<0.0001**	0.24	**0.02**	**0.0003**
Small	277	30.25±0.07 °C^{A}	24.00±0.18°C	3.32±0.05°C^{AB}	5.72±0.08°C^{B}
Medium	33	30.37±0.20°C^{A}	23.55±0.33°C	3.02±0.14°C^{B}	5.44±0.22°C^{B}
Large	96	29.67±0.12°C^{B}	23.71±0.19°C	3.48±0.08°C^{A}	6.25±0.13°C^{A}
Season placed (P-value)		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Autumn	108	29.66±0.08°C^{C}	22.31±0.12°C^{D}	2.98±0.06°C^{C}	5.09±0.10°C^{B}
Winter	93	29.50±0.09°C^{C}	22.80±0.12°C^{C}	2.74±0.06°C^{D}	5.28±0.11°C^{B}
Spring	159	30.32±0.07°C^{B}	25.16°0.10°C^{B}	3.64±0.05°C^{B}	6.57±0.09°C^{A}
Summer	46	32.26±0.15°C^{A}	26.19±0.20°C^{A}	4.63±0.10°C^{A}	6.37±0.18°C^{A}
Presence/absence of the APS * Shed design (P-value)		0.86	0.52	0.21	0.26
APS present and new shed	23	30.01±0.24°C^{AB}	24.80±0.39°C^{A}	4.11±0.17°C^{A}	6.45±0.26°C^{A}
APS present and old shed	6	30.80±0.51°C^{AB}	24.76±0.83°C^{AB}	3.58±0.36°C^{AB}	6.45±0.55°C^{AB}
APS absent and new shed	73	29.57±0.13°C^{B}	23.36±0.22°C^{B}	3.28±0.09°C^{B}	6.19±0.15°C^{A}
APS absent and old shed	304	30.26±0.07°C^{A}	23.94±0.11°C^{AB}	3.28±0.05°C^{B}	5.67±0.07°C^{B}
Presence/absence of the APS * Flock size (P-value)		0.34	0.72	0.33	0.49
APS present and Large	23	30.01±0.24°C^{AB}	24.80±0.39°C^{A}	4.11±0.17°C^{A}	6.45±0.26°C^{AB}
APS present and Medium	1	29.42±1.14°C^{AB}	23.49±1.86°C^{AB}	2.73±0.80°C^{AB}	6.17±1.24°C^{ABC}
APS present and Small	5	31.15±0.57°C^{AB}	25.08±0.93°C^{AB}	3.80±0.40°C^{AB}	6.77±0.62°C^{ABC}
APS absent and Large	73	29.57±0.13°C^{B}	23.36±0.22°C^{B}	3.28±0.09°C^{B}	6.19±0.15°C^{A}
APS absent and Medium	32	30.40±0.20°C^{A}	23.55±0.33°C^{AB}	3.03±0.14°C^{B}	5.41±0.22°C^{C}
APS absent and Small	272	30.24±0.07°C^{A}	23.98±0.12°C^{AB}	3.31±0.05°C^{B}	5.70±0.08°C^{BC}

Variable (effect and level)	**Number of flocks (n)**	**Mean maximum shed temp. 0-25 days of age**	**Mean maximum shed temp. 26-51 days of age**	**Mean fluctuation in shed temp. 0-25 days of age**	**Mean fluctuation in shed temp. 26-51 days of age**
Presence/absence of the APS		0.07	0.14	**0.009**	**0.01**
* Season placed (P-value)					
APS present and Autumn	2	$28.0\pm0.6°C^{D}$	$21.1\pm0.8°C^{C}$	$2.77\pm0.41°C^{CD}$	$6.43\pm0.75°C^{ABC}$
APS present and Winter	4	$29.3\pm0.4°C^{CD}$	$23.9\pm0.6°C^{BC}$	$2.76\pm0.29°C^{CD}$	$6.80\pm0.53°C^{AB}$
APS present and Spring	20	$30.4\pm0.2°C^{BC}$	$25.2\pm0.3°C^{AB}$	$4.33\pm0.13°C^{A}$	$6.42\pm0.24°C^{A}$
APS present and Summer	3	$31.9\pm0.6°C^{AB}$	$25.9\pm0.1°C^{AB}$	$4.56\pm0.41°C^{AB}$	$6.57\pm0.75°C^{ABC}$
APS absent and Autumn	106	$29.7\pm0.1°C^{D}$	$22.3\pm0.1°C^{C}$	$2.98\pm0.06°C^{D}$	$5.06\pm0.10°C^{C}$
APS absent and Winter	89	$29.5\pm0.1°C^{D}$	$22.8\pm0.1°C^{C}$	$2.74\pm0.06°C^{D}$	$5.20\pm0.11°C^{BC}$
APS absent and Spring	139	$30.3\pm0.1°C^{BC}$	$25.1\pm0.1°C^{B}$	$3.54\pm0.05°C^{BC}$	$6.59\pm0.09°C^{A}$
APS absent and Summer	43	$32.3\pm0.2°C^{A}$	$26.2\pm0.2°C^{A}$	$4.63\pm0.10°C^{A}$	$6.36\pm0.18°C^{A}$

Table 5.7 outlines the OR associated with unit increases and decreases (where relevant) in mean weekly maximum shed temperature readings beyond the Ross 308 standard shed temperature guidelines (Aviagen, 2012) and the presence of the APS. The units for each week were derived from one standard deviation in the means for each week in the dataset. It is evident that the age at which the highest risk of the APS developing from increases in mean maximum shed temperature above the standard guidelines was 29-35 days of age, at which point each unit increase in temperature above the guidelines was associated with 1.96 times greater odds of the APS developing in a flock. Additionally, it was evident that shed temperature increases above the Ross 308 standards had a protective effect in the period placement-7 days as the 95% confidence interval was below unity. Thus subsequent analysis showed that with each unit decrease in mean maximum shed temperature below the Ross 308 standards in the period placement – 7 days of age flocks incurred 3.26 greater odds of developing the APS.

Table 5.7 Odds ratios associated with weekly mean maximum shed temperature deviations from the standard Ross 308 shed temperature guidelines (Aviagen, 2012) and the presence of the APS

Age (days)	**Mean standard whole shed temp. (°C).**	**Mean maximum (one standard deviation shed temp. (°C)**	**OR (95% CI) of APS per standard deviation increase above guidelines**	**P-value**
1-7	28.3	33.4 (1.0)	0.31 (0.17, 0.52)	**<0.0001**
8-14	25.8	30.2 (1.2)	0.83 (0.54, 1.22)	0.34
15-21	23.2	28.7 (1.7)	**1.49** (1.02, 2.17)	**0.04**
22-28	21.1	26.8 (1.7)	1.47 (0.99, 2.26)	0.06
29-35	20.0	24.6 (1.8)	**1.96** (1.28, 3.14)	**0.002**
36-42	20.0	23.3 (2.1)	**1.49** (1.01, 2.25)	**0.04**
43-49	20.0	22.9 (2.3)	1.30 (0.86, 1.98)	0.21
Age (days)	**Mean standard whole shed temp. (°C).**	**Mean maximum (one standard deviation shed temp. (°C)**	**OR (95% CI) of APS per standard deviation decrease below guidelines**	**P-value**
1-7	28.3	33.4 (1.0)	**3.26** (1.91, 5.90)	**<0.0001**

5.3.3.6 External temperature (Bureau of Meteorology)

Daily maximum and minimum temperatures for the specific region for the period encompassing the data set were obtained from the Australian Government Bureau of Meteorology (National Climate Centre, 2014) and from such daily fluctuations in temperature were derived. Figure 5.9 plots the mean daily maximum external temperatures through the age of flocks by the presence/absence of the APS. Repeated measures analysis showed significant effects of both day ($P < 0.0001$) and the presence or absence of the APS ($P < 0.0001$) highlighting that maximum external temperatures varied significantly throughout the age of the flock and also between flocks with and without the APS. Additionally the effect on maximum external temperatures from the interaction between day and the presence/absence of the APS was also significant ($P < 0.0001$), thus confirming that variations in maximum external temperatures between flocks with and without the APS varied over time. Background variation between flocks accounted for 60.28 % of

this variation (variance ratio = 1.52), consistent with flocks being placed at different times throughout the year.

The mean daily maximum external temperatures were significantly greater (P = 0.0002) associated with flocks with the APS (27.3±1.0°C) compared to flocks without the APS (23.3±0.3°C) (Figure 5.9). These findings were consistent with the greater proportion of flocks with APS being placed in warmer periods of the year (Figure 5.1). Univariate logistic analysis found that for each unit increase (5.6°C) in mean daily maximum external temperature through the life of a flock the odds of the APS developing increased 2.14 (1.43, 3.30) times (Table 5.11).

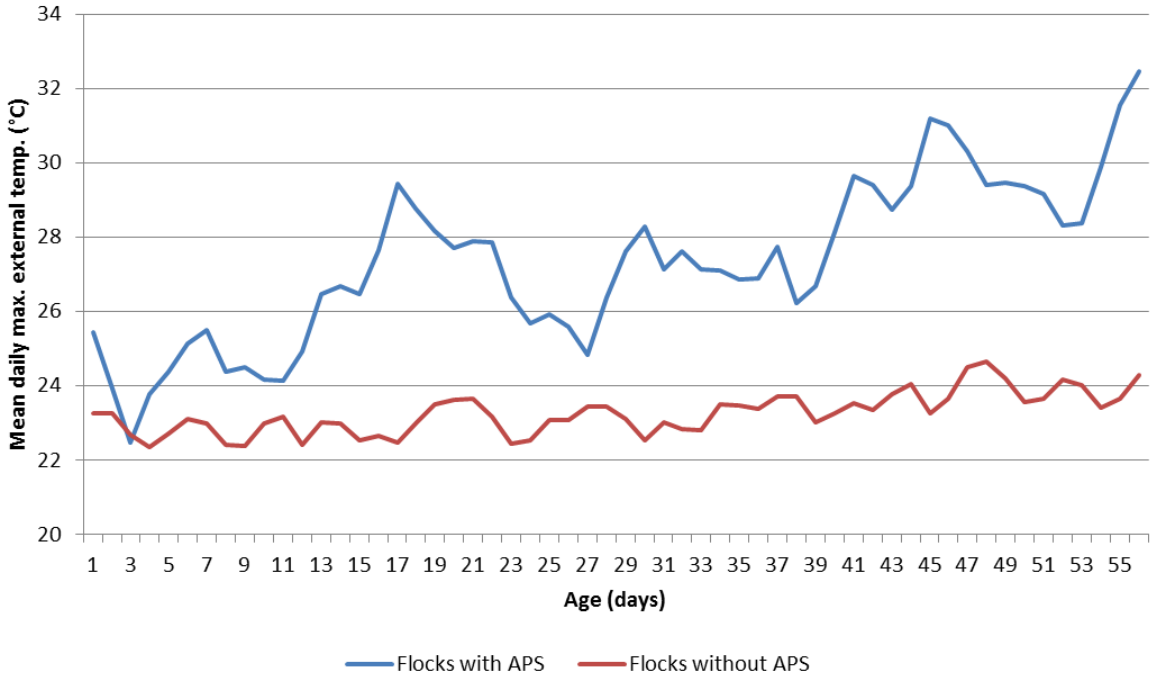

Figure 5.9 Mean daily maximum external temperatures associated with flocks with (blue line) and without (red line) the APS.

Figure 5.10 plots the mean daily maximum external temperature fluctuations as a function of age, stratified by the presence/absence of the APS. Repeated measures analysis showed significant effects of both day (P < 0.0001) and the presence or absence of the APS (P < 0.0001) highlighting that mean external temperature fluctuations varied significantly throughout the age of the flock and also between flocks with and without the APS. Additionally the effect on mean external temperature fluctuations from the interaction between day and the presence or absence of the APS was also significant (P < 0.0001), thus confirming that variations in mean daily external shed temperature fluctuations between flocks with and without the APS were time variable. Background variation between flocks accounted for 18.01 % of this variation (variance ratio 0.2).

The mean daily fluctuations in external temperature were also significantly greater (P = 0.003) associated with flocks with the APS (15.59±0.38°C) compared to flocks without the APS (14.43±0.10°C) (Figure 5.10). Univariable logistic analysis found that for each unit increase (2.1°C) in mean daily external temperature fluctuations through the life of a flock the odds of the APS developing increased 1.78 (1.22, 2.70) times (Table 5.11).

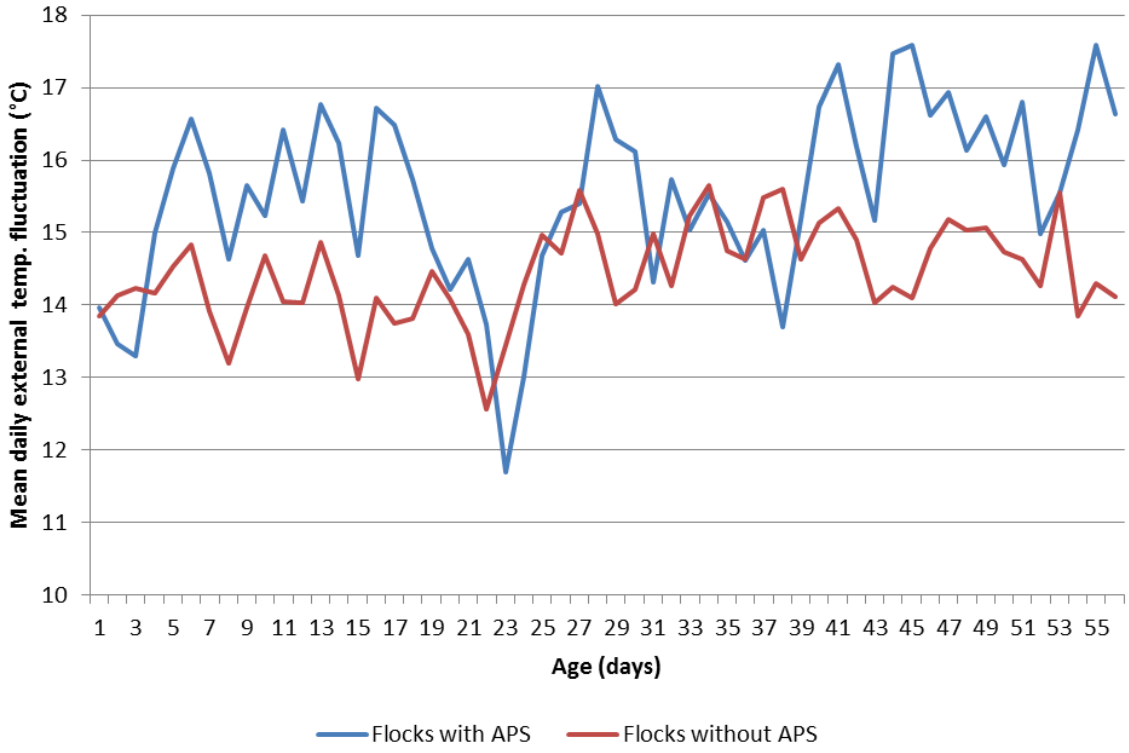

Figure 5.10 Mean daily fluctuations in external temperatures associated with flocks with (blue line) and without (red line) the APS.

5.3.3.7 Stocking density at placement

Stocking density data were derived from chicken placement and shed dimension data. There was not any variation in stocking density by presence/absence of the APS, sex status, shed design or flock size. Flocks were placed at greater density in autumn and winter compared to spring and summer (Table 5.8). Within sexed flocks there was a significant difference in stocking density at placement between males and females, with females being placed at a greater density than males (Table 5.8) which was a result of the practice adopted in the region of removing more females at the earlier thin-out time.

Table 5.8 Summary data in relation to stocking density, results with a different letter are significantly different

Test	Result	P-value
Presence/absence of the APS		0.97
Sex status		0.76
Shed design		0.13
Flock size		0.80
Season placed		**< 0.0001**
Autumn	18.29±0.06 chicks/$m^{2\ A}$	
Winter	18.05±0.07 chicks/$m^{2\ B}$	
Spring	17.67±0.05 chicks/$m^{2\ C}$	
Summer	17.48±0.10 chicks/$m^{2\ C}$	
Placement density within sexed flocks		**P < 0.0001**
Males	16.78±0.10 chicks/$m^{2\ B}$	
Females	19.60±0.10 chicks/$m^{2\ A}$	

5.3.3.8 Parent flocks and hatchery

In total 245/406 (60.3%) of broiler flocks consisted of chickens sourced from multiple parent flocks (MP) and 161/406 (39.7%) from a single parent flock (SP). The incidence of the APS in MP broiler flocks was significantly greater than for SP broiler flocks (P = 0.01) with 24/245 MP broiler flocks having the APS compared to 5/161 SP broiler flocks. MP flocks experienced 3.43 (1.38, 10.35) greater odds of developing the APS than did SP flocks (Table 5.11). However, this result was found to have been confounded by flock size (small, medium or large) as the Cochran-Mantel-Haenszel test showed the relationship between the APS and parent flock (MP or SP) to be non-significant (P = 0.13) when the data were stratified by flock size at placement and reflects the need to source chickens from multiple parent flocks to fill larger shed placements.

Table 5.9 outlines the source hatcheries for the 406 flocks placed in the data set. There was not any association (P = 0.21) between the presence of the APS and flocks being supplied by single or multiple hatcheries. The significance was even less (P = 0.40) when a Cochran-Mantel-Haenszel test was performed with data being stratified by flock size.

Table 5.9 Summary of hatchery supply of flocks in data set

Hatchery	Proportion of total flocks	APS in flocks
A	310/406 (76.4 %)	21/310 (6.8 %)
B	18/406 (4.4 %)	0/18 (0 %)
C	14/406 (3.4 %)	1/14 (7.1 %)
A + C	58/406 (14.3 %)	7/58 (12.1 %)
A + B	4/406 (1.0 %)	0/4 (0 %)
A + B + C	2/406 (0.5 %)	0/2 (0 %)

5.3.3.9 Scheduled thin-outs for processing

Thin-outs involved removal of a portion of the flock for processing at stages throughout the growing period which were set by market requirements and performance of the flock at that time. Flocks were typically thinned-out on up to three occasions prior to final processing of the flock. Table 5.10 provides the mean flock age at each thin-out along with the OR of the APS developing with each unit increase in flock age for each thin-out. It was evident that the timing of the first thin-out was significant in that each age unit increase in flock age at the 1st thin-out was associated with 1.41 greater odd of the APS developing in that flock.

Table 5.10 Univariate analyses of thin-out times in relation to the presence/absence of the APS

Thin-out	Mean flock age (one standard deviation) at thin-out (days)	OR (95% CI) of APS per standard deviation increase	P-value
1st	35 (1.2)	**1.41** (1.04, 1.90)	**0.03**
2nd	38 (2.6)	1.43 (1.00, 1.97)	0.05
3rd	47 (2.7)	0.91 (0.57, 1.53)	0.70

5.3.3.10 Summary of individual risk factors for the APS

The data set out in Table 5.11 summarise the results in descending OR order from univariate logistic regression analysis in relation to the presence or absence of the APS in a flock (dependant variable) with respect to each of the flock variables discussed earlier in the previous two sections. Farm cluster location was excluded from these analyses given the absence of the APS on farms in cluster A. Univariate logistic regression analysis forms the 1st step of the multivariate LRM which is presented in 5.3.4.

Table 5.11 Results from univariate logistic regression analysis in order of descending odds ratios

Risk factors associated with the presence of the APS	Est. Coef.	Std. Error	Wald ChiSq	P-value	OR (95% CI)
Shed design (old = 0, new = 1)	1.39	0.24	33.79	< **0.0001**	**15.96** (6.66, 44.50)
Spring placement (other = 0, spring = 1)	0.67	0.21	11.36	**0.0008**	**3.80** (1.73. 9.00)[1]
Flock size (1 unit increase = 10,827 birds)	1.23	0.21	34.37	< **0.0001**	**3.44** (2.34, 5.40)
Parent flock source (single = 0, multiple = 1)	0.62	0.25	6.00	**0.01**	**3.43** (1.38, 10.35)
Mean daily maximum shed temperature decrease below Ross 308 standards (placement – 7 days of age) (1 unit increase = 1.0°C)	1.18	0.29	16.92	<**0.0001**	**3.26** (1.91, 5.90)
Mean flock bodyweight above Ross 308 standards: 14 days of age (1 unit increase = 34 g)	0.82	0.21	15.36	< **0.0001**	**2.26** (1.52, 3.46)
Sex status of flock (AH = 0, sexed = 1)	-0.40	0.20	4.25	**0.04**	**2.24** (1.05, 4.94)
Mean flock bodyweight above Ross 308 standards: 21 days of age (1 unit increase = 66 g)	0.79	0.20	15.51	< **0.0001**	**2.20** (1.50, 3.29)
Mean daily shed temperature fluctuation (placement – 25 days of age) (1 unit increase= 0.8°C)	0.79	0.18	18.15	< **0.0001**	**2.19** (1.54, 3.19)
Mean daily maximum external temperature (1 unit increase = 5.6°C)	0.76	0.21	12.78	**0.0003**	**2.14** (1.43, 3.30)
Mean daily shed temperature fluctuation (26-51 days of age) (1 unit increase = 1.3°C)	0.68	0.24	7.71	**0.006**	**1.97** (1.25, 3.29)
Mean daily maximum shed temperature increase above Ross 308 standards (29-35 days of age) (1 unit = 1.8°C)	0.67	0.22	8.73	**0.002**	**1.96** (1.28, 3.14)
Mean flock bodyweight above Ross 308 standards: 28 days of age (1 unit increase = 89 g)	0.62	0.19	10.20	**0.001**	**1.86** (1.28, 2.76)
Hatchery (single = 0, multiple = 1)	0.30	0.23	1.73	0.21	1.82 (0.69, 4.28)
Mean daily external temperature fluctuation (1 unit increase = 2.1°C)	0.58	0.20	8.52	**0.003**	**1.78** (1.22, 2.70)
Mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age) (1 unit increase = 1.7°C)	0.40	0.19	4.37	**0.04**	**1.49** (1.02, 2.17)
Mean daily maximum shed temperature increase above Ross 308 standards (36-42 days of age) (1 unit increase = 2.1°C)	0.40	0.20	3.89	**0.04**	**1.49** (1.01, 2.25)
Mean daily maximum shed temperature increase above Ross 308 standards (22-28 days of age) (1 unit = 1.7°C)	0.39	0.21	3.43	0.06	1.47 (0.99, 2.26)
Timing of 2nd thin-out (1 unit increase = 2.6 days)	0.36	0.17	4.39	0.05	1.43 (1.00, 1.97)
Timing of 1st thin-out (1 unit increase = 1.2 day)	0.34	0.15	4.97	**0.03**	**1.41** (1.04, 1.90)
Mean flock bodyweight above Ross 308 standards: 35 days of age (1 unit increase = 105 g)	0.34	0.20	2.89	0.09	1.40 (0.95, 2.07)
Mean daily maximum shed temperature increase above Ross 308 standards (43-49 days of age) (1 unit increase= 2.3°C)	0.26	0.21	1.56	0.21	1.30 (0.86, 1.98)
Mean flock bodyweight above Ross 308 standards: 7 days of age (1 unit increase = 14 g)	0.16	0.20	0.63	0.42	1.17(0.80, 1.77)
Density placed (1 unit increase = 0.72 chicks/m^2)	-0.06	0.19	0.11	0.74	0.94 (0.66, 1.39)
Timing of 3rd thin-out (1 unit increase = 2.7 days)	-0.10	0.25	0.15	0.70	0.91 (0.57, 1.53)
Summer placement (other = 0, summer = 1)	0.05	0.32	0.03	0.86	0.90 (0.21, 2.67)[1]
Winter placement (other = 0, winter = 1)	0.33	0.28	1.42	0.20	0.52 (0.15, 1.38)[1]
Mean daily maximum shed temperature increase above Ross 308 standards (8-14 days of age) (1 unit increase= 1.2°C)	-0.19	0.20	0.87	0.34	0.83 (0.54, 1.22)

[1]Design variables derived using reference cell coding with autumn as the reference group.

5.3.4 Multivariate logistic regression analysis

The results from univariate logistic regression analyses presented in Table 5.11 represent the first step required in multivariate logistic regression analysis. From step 1, independent variables with $P < 0.25$ from Table 5.11 were retained for step 2 which required each to be entered into a multivariate model. From step 2 the independent variables which were retained included: mean daily maximum shed temperature decrease below Ross 308 standards (placement – 7 days of age), flock size, mean daily shed temperature fluctuation (placement – 25 days of age), mean daily shed temperature fluctuation (26-51 days of age), mean daily maximum external temperature, mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age) and timing of 2nd thin-out. From step 3 the following interactions were retained for step 4: mean daily shed temperature fluctuation (placement – 25 days of age) * mean daily maximum external temperature, mean daily shed temperature fluctuation (placement – 25 days of age) * mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age) and mean daily maximum external temperature * mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age). From step 4 the following were retained for step 5: mean daily maximum shed temperature decrease below Ross 308 standards (placement – 7 days of age), flock size, mean daily maximum external temperature, mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age) and the interaction between mean daily maximum external temperature * mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age). The results from step 5 are shown in Table 5.14.

Table 5.14 Results from final multivariate logistic regression model of predictive factors for the APS

Variable / interaction	**Est. Coef.**	**Std. Error**	**Odds ratio**	**95% confidence interval**	**Wald chi-square**	**P-value**
Mean daily maximum shed temperature decrease below Ross 308 standards (placement – 7 days of age) (1 unit = 1.0°C)	-5.84	2.62	**3.99**	1.73, 10.11	9.60	**0.002**
Flock size (1 unit increase = 10,827 chickens)	1.38	0.45	**3.00**	1.80, 5.51	15.23	**<0.0001**
Mean daily maximum external temperature (1 unit increase = 5.6°C)	1.01	0.50	**2.73**	1.11, 8.10	4.09	**0.04**
Mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age) (1 unit = 1.7°C)	-0.03	0.52	0.97	0.33, 2.61	0.003	0.96
Mean daily maximum external temperature (1 unit increase = 5.6°C) * Mean daily maximum shed temperature increase above Ross 308 standards (15-21 days of age) (1 unit = 1.7°C)	0.82	0.28	n/a	n/a	8.39	**0.004**

n = 386, $r^2 = 0.42$. Hosmer-Lemeshow test: P = 1.00, df = 5.

From the final multivariate LRM it was evident that shed temperatures below the Ross 308 standards in the first week of life, larger flock size and higher external temperatures were highly associated with the presence of the APS. For each unit decrease (1.0°C) in mean daily maximum shed temperature below the Ross 308 whole shed temperature guidelines from placement – 7 days of age there were 3.99 greater odds of a flock developing the APS. For each unit increase in flock size (10,827 chickens) there were 3.00 greater odds of a flock developing the APS. For each unit increase (5.6°C) in mean maximum external temperature through the life of a flock there were 2.73 greater odds of a flock developing the APS. Additionally, the interaction between mean daily maximum external temperature through the life of a flock and the mean daily maximum shed temperature increase above the Ross 308 guidelines through 15-21 days of age had a significant effect in the final model. This effect is shown in Figure 5.11 which shows that increases in external temperature were associated with significantly greater increases in maximum shed temperatures above the Ross 308 standard in the period 15-21 days of age in flocks with the APS compared to flocks without the APS.

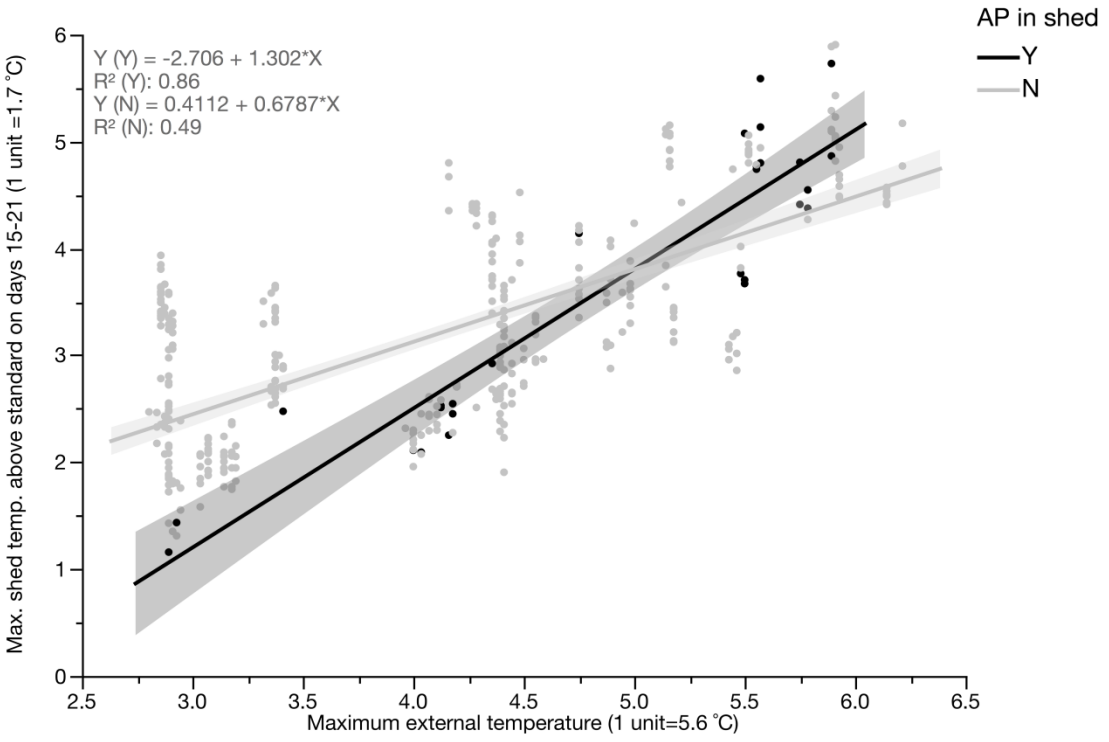

Figure 5.11 Interaction plot showing mean maximum external temperature (1 unit = 5°C) on the x-axis and mean maximum shed temperature above the Ross 308 standard (15-21 days of age) (1 unit = 2.03°C) on the y-axis by presence/absence of the APS. Each dot represents one flock (n=406) and shaded areas represent the 95% confidence interval.

5.4 Discussion

The analyses presented in this chapter were unfortunately restricted to a relatively short period of time which was due to the availability of suitable data and time restrictions in completing the analyses. Data were only used from flocks for which daily records were held and for this reason not all cases of APS in the region during the sampling period were included in these epidemiological analyses. Moving forward, as data recording practices improve, such should allow for longer term and potentially more useful analyses. The method used to define a case of the APS was considered to be accurate given the unique clinical appearance of broilers affected with the APS (Chapter 4).

5.4.1 Description of the APS: temporal and spatial distribution

The most striking feature from the analysis of the spatial distribution of the APS was that two farms (farms 13 and 14) accounted for 79 % of all cases of the APS. These two farms also accounted for all new design sheds and had the largest flock placement sizes. I relied on the multivariate analyses reported in order to investigate what it was about these two farms that explained their relatively high incidence of the APS.

There was a clear trend observed over the study period of the increased incidence of flocks with APS placed in spring and summer, from a peak incidence of 32 % of flocks placed developing the APS in (spring) to a trough of just 1 % of flocks placed in autumn developing the APS (Figure 5.1). This general trend was confirmed by the univariate analyses, which demonstrated significant odds of the APS developing in flocks placed in spring (OR 3.80) and non-significant odds for summer and winter placed flocks (OR 0.90 and 0.51 respectively) compared to Autumn (Table 5.11). These findings were consistent with findings in relation to maximum shed and external temperatures (to be discussed), as for spring placed flocks at peak mortality (26-51 days of age) temperatures would be expected to be highest.

5.4.2 Analysis of the effect of the APS on broiler flock performance

The mean weekly bodyweights were higher in flocks with the APS than flocks without the APS at 14, 21 and 28 days of age. Within each sex status, bodyweights of AH flocks did not vary by the presence/absence of the APS at any age, whereas the male portion of sexed flocks were significantly heavier in those flocks with the APS compared to flocks without the APS at 14, 21, 28, 35, 42 and 49 days of age. The same finding was only observed in female portions of sexed flocks at 14 days of age. The similarity in weights in AH flocks with and without the syndrome is likely a result of relatively larger proportions of sexed flocks being placed on farms 13 and 14

which accounted for the larger proportion of cases of the APS. This is consistent with bodyweights being significantly greater in flocks in new as opposed to old sheds and large as opposed to small and medium sized flocks at all ages measured as all new shed placements and large flocks were on farms 13 and 14. Bodyweights were typically greater in flocks placed in spring and summer compared to autumn and winter at 21, 28 and 35 days of age. This is consistent with a greater proportion of flocks placed in spring and summer developing the APS compared to autumn and winter placed flocks (Figure 5.1). Whilst the APS did not deleteriously affect the growth performance of broiler flock it did results in significant losses from mortality and culls.

5.4.3 Risk factors for the APS

Univariate analyses provided indications as to the importance of individual risk factors in relation to the presence of the APS and Table 5.11 lists each factor in order of descending OR. However, this approach alone does not provide a completely satisfactory means to assess the relative importance of each factor, particularly in this case as we have identified the potential for confounding of the factors of flock size, shed design and sex status by farm and of parent flock source by flock size. Multivariate logistic regression models offer a flexible way of investigating the risk factors for disease when the included factors can represent causative factors and/or confounders of the disease-causation relationship (McNamee, 2005). Thus whilst univariate analyses represent an essential first step in identifying potential risk factors for the APS, the multivariate approach is essential in order to define the relative importance of the different factors and thus to identify the most useful corrective actions which may be implemented to reduce the incidences of the APS.

Univariate logistic regression analysis based on SDU increases in weekly bodyweight above the Ross 308 standard performance guidelines showed significant risk of the APS developing with increases in bodyweights above the guidelines at 14, 21 and 28 days of age for all flocks; 14, 21, 28 and 35 days of age for males; and 14 and 21 days of age for females. The highest odds associated with elevated bodyweights and the presence of the APS was detected at 14 days of age for all flocks (OR 2.26), males (OR 3.88) and females (2.09). These results provide direction for further investigation as to whether the incidence of the APS could be reduced by restricting growth in the first 14 days of age to the level of or below the Ross 308 standard performance guidelines.

Diseases to which better performing animals are more susceptible are rare. Relevant to chickens, there is believed to be a weak but positive correlation between pulmonary hypertension syndrome

(ascites) and higher bodyweight (Crespo & Shivaprasad, 2008). In pigs, the porcine stress syndrome (PSS), due to mutations in the ryanodine receptor 1 gene, which results in a muscle rigidity, a rapid tremor of the tail, dyspnoea and acute death following episodes of stress has a high incidence in strains selected for high growth rates, heavy muscling and efficient feed conversion (Blood *et al.,* 1979). In ruminants, enterotoxaemia is a disease which affects rapidly growing animals and is caused by the epsilon-toxin from *Clostridium perfringens* type D. Affected animals die suddenly but prior to death and can exhibit ataxia, stiffness, convulsions, opisthotonus and coma (Michelsen & Smith, 2009). The disease occurs when conditions within the gastrointestinal tract favour the over proliferation of *C. perfringens,* which results in the presence of large amounts of the prototoxin, which once cleaved increases intestinal permeability and induces oedema in a wide variety of organs. Excess fluid is often seen in body cavities and perivascular oedema is often noted in the brain; however, gross lesions in the gastrointestinal tract are typically not observed. *C. perfringens* can cause disease (necrotic enteritis) in poultry due to NetB (Keyburn *et al.,* 2008) and beta-toxins released from the organism. The toxins result in intestinal necrosis which is the characteristic lesion of the disease (Opengart, 2008). Flocks affected with *C. perfringens* disease typically show a reduction in performance and brain histopathology is not reported, which clearly differentiates this disease from the APS. Although a syndrome similar to enterotoxaemia as it occurs in ruminants has not been reported in chickens, the possibility of such requires further consideration in relation to the APS. Obvious differences between enterotoxaemia as it occurs in ruminants and the APS in chickens are the absence of encephalitis in enterotoxaemia, the absence of gross pathology in chickens with the APS and and the absence of hyperglycaemia in broilers with the APS. The reported reference range for blood glucose levels in broiler chickens are 7.6 - 20.1 mmol/L (Goodwin *et al.,* 1994), with measurements from broilers with the APS falling within this range (Chapter 4). ELISA kits specific to the *C. perfringens* type D epsilon toxin are available and this could be a useful step in this investigation in order to rule out *C. perfringens* type D - induced enterotoxaemia as a differential diagnosis for the APS.

There were significant associations between the presence of the APS and flocks placed in new as opposed to old sheds (OR 15.96), flocks placed sexed as opposed to AH (OR 2.24) and larger flock sizes (OR 3.44 per unit increase of 10,827 chickens) (Table 5.11). All of these effects were confounded with the effect of farm ID as farms 13 and 14 which accounted for 79% of all flocks with the APS contained the only new sheds and all of the large flock placements in the dataset and had a high proportion of flocks placed sexed. From the multivariate LRM (Table 5) flock size was shown to be highly associated ($P < 0.0001$) with the presence of the APS and with each SDU

increase in flock size (10,827 chickens) the odds of the APS developing was increased 3.00 times. The factors of shed design and flock sex status were relatively unimportant from the multivariate LRM. Whilst the importance of flock size as a risk factor for the APS is interesting, it is unrealistic to consider reducing flock sizes using existing farm infrastructure given the investment associated with setting up modern day farms and the production levels required to achieve adequate returns on investment. If the APS was caused by an infectious agent/s, the importance of flock size as a risk factor for the APS could be explained by larger flock sizes achieving greater and faster amplification and transmission of the agent/s.

Although there was a non-significant difference between the mean daily maximum shed temperatures through the whole period placement – 25 days of age, there was a high level of risk of the APS developing from SDU deviations below the Ross 308 whole shed temperature guidelines in the period placement to 7 days of age with the odds of the APS increasing by 3.26 with each SDU (1.0°C) decrease (Table 3). The multivariate LRM found this to be the most important risk factor as determined by an OR of 7.60 in the final model for each SDU decrease below the guidelines. Thus it is recommended that closer monitoring and regulation of shed temperatures in the first week be implemented in order to reduce incidences of the APS.

At the time of peak mortality (26-51 days of age) for flocks with the APS, the mean daily maximum shed temperature was significantly greater than for flocks without the APS. Having mean maximum shed temperatures above the Ross 308 standard whole shed temperature guidelines through 15-21, 29-35 and 36-42 days of age therefore posed significant risk for APS with the odds of the APS developing increasing by 1.49, 1.96 and 1.49 respectively for each SDU increase (Table 5.11). These findings were consistent with the incidence of the APS being higher in flocks placed in spring and summer (Figure 5.1) when shed temperature would be expected to be highest despite ventilation efforts. The significance of this relationship was highlighted in the multivariate LRM, as the maximal shed temperature increased more drastically as the external temperature increased in flocks with the APS than in flocks without the APS (Figure 5.11). Thus it is recommended that closer monitoring and regulation of ventilation strategies to cool birds later in life if the shed temperatures exceed the guidelines be enforced in order to reduce the incidence APS.

Mean daily shed temperature fluctuations were found to be significantly greater for flocks with the APS compared to flocks without the APS in both periods (0– 25 and 26-51 days of age). Univariate analysis showed that SDU increases in mean daily shed temperature fluctuations through placement - 25 and 26-51 days of age were associated with 2.19 and 1.97 greater odds of

the APS developing (Table 5.11). It is possible that these finding are linked to delayed corrective responses to elevations in maximum shed temperatures as previously discussed.

Daily maximum and fluctuations in external temperatures were significantly greater associated with the flocks with the APS compared to flocks without the APS through the life of each flock and the odds of the APS developing in a flock increased by 2.14 and 1.78 respectively for each unit increase (Table 5.11) from univariate analyses. The result in relation to maximum temperature was consistent with the increased incidence of flocks with the APS placed at warmer periods of the year (Figure 5.1). Understanding these trends is important in terms of implementing monitoring and corrective strategies in order to minimise the effect external temperature has on shed temperatures in relation to controlling the APS.

A significant difference was detected in the mean age at second thin-out with flocks with the APS being significantly older than flocks with the APS. Mortality rates in flocks with the APS had typically already began to spike by the time of the second thin-out (Figure 5.2). It is conceivable that higher stocking densities associated with delayed thin-out could contribute to higher maximum shed temperatures observed in flocks with the APS.

5.5 Conclusion

The APS results in significant levels of wastage in affected flocks which is of economic and welfare importance. This chapter provides indications as to further areas of investigation which could be implemented in the region in order to reduce the incidence of the APS. From the analyses presented, the most important factor in order to minimise the incidence of the APS is to achieve greater control of brooding shed temperatures in the first week of life. Other strategies which may be considered include: closer monitoring and control of maximum shed temperatures from 15 days of age, maintenance of more consistent shed temperatures through the life of the flock, maintenance of bodyweights at the Ross 308 guidelines particularly in the first 14 days of age and tighter control of thin-out times.

Chapter 6: Experimental reproduction of an acute paralysis syndrome of broiler chickens

6.1 Introduction

The acute paralysis syndrome (APS) previously described is characterised by flaccid paralysis of the neck of affected chickens and elevated mortalities in flocks from 26 – 51 days of age (Chapter 5). In a proportion of affected chickens, vasculocentric inflammatory pathology at the base of the cerebrum and Wallerian degeneration in the spinal cord has been observed (Chapter 4). Mortality increased more in the male than in the female portion of flocks affected with the APS, suggesting that males were more susceptible to the APS (Chapter 5). Vaccination of broilers with herpesvirus of turkeys (HVT) has not prevented the APS (Chapter 4). ND, avian influenza (AI) and botulism have been ruled out as differential diagnoses for the APS (Chapter 4). Given that the APS has features consistent with known viral infections of poultry, it is possible that the APS may be reproduced by challenging chickens with material (tissue and/or environmental) harvested from field cases of the APS in the presence of an infectious aetiological agent(s). The development of an effective experimental reproduction model is thus an essential component of further investigations into the causation of the APS. It was highlighted in Chapter 4 that future investigations should specifically test chickens affected with the APS for the presence of Marek's disease virus serotype 1 (MDV1), avian encephalomyelitis virus (AEV) and flaviviruses is required.

Farms affected with the APS did not re-use shed litter between batches of chickens and sheds were formaldehyde fumigated between batches (Chapter 5). This suggests that exposure to any infective agent that may be associated with the APS would be delayed. It is possible that this delay may coincide with the waning of maternal immunity that may be present to an infectious agent as the protective effects of maternal antibody (MAb), typically decay below protective levels from 14 days of age (Patterson *et al.,* 1962). Specific pathogen free (SPF) chickens are considered free of MAb against a wide-range of infectious agents and thus provide an ideal means for the study of an infectious disease in the absence of MAb. Thus the use of SPF chickens and multiple ages of challenge in broiler chickens are essential features of early experimental reproduction attempts for the APS to explore the potential involvement of delayed exposure to an infectious agent in the pathogenesis of the APS.

This experiment, using commercial Ross 308 broiler and SPF chickens was designed with the following objectives:

- To reproduce and clinically describe the APS;
- To determine modes of transmission for the potential infectious agent/s involved in the APS;
- To confirm that vaccination with HVT does not afford protection against the APS in broiler chickens;
- To determine whether the age of exposure to potentially infectious material affects the manifestation of the APS; and
- To harvest tissue to assist with screening for and possible isolation of infectious agents (including specifically MDV1, AEV and flaviviruses), histopathological evaluation and further passage.

The experiment was designed to test the following specific hypotheses:

1. That the APS is transmissible between chickens as determined by its observation in Ross broiler chickens inoculated with tissue from affected chickens or exposed to shed litter from affected sheds; and not in sham treated chickens.
2. That MDV is not the causative agent of the APS, as determined by *in ovo* HVT vaccinated chickens remaining susceptible to the APS;
3. That susceptibility varies with age or MAb status, with day old chickens being less susceptible than those at 21 days of age at challenge age.

6.2 Materials and Methods

6.2.1 Experimental Design

This experiment (13-C-REP1) was approved by the University of New England (UNE) Animal Ethics Committee (AEC13-024) and ran for a total of 56 days (commenced 8th April and terminated 5th June 2013). It had a 2 x 2 x 2 x 5 factorial design as set out in Table 6.1. The four experimental factors were:

- Chicken type: two levels were used being Ross broiler chickens and SPF chickens. The SPF chickens were hatched and placed on day -5 of the experiment, and the Ross broiler chickens were hatched and placed on day 0 of the experiment.
- Vaccination status: two levels were used for broiler chicken groups, with equal numbers of HVT (Vaxsafe® HVT, Bioproperties Pty Ltd., Batch 1108) vaccinated (*in-ovo,* day 18 of

incubation) chickens and unvaccinated chickens being placed. All SPF chickens used were unvaccinated.

- Age at challenge: two levels were used, with broiler and SPF chickens being challenged at either day 0 (Expt d0) or day 21 (Expt d21) of the experiment.
- Challenge material: five levels were used, which included sterile cell culture medium (buffer) for sham treatment, spleen cells harvested from affected birds from a field outbreak, brain harvested from the same birds, whole blood harvested from the same birds and shed litter collected from the same field outbreak.

The sham-challenged SPF chickens (Table 6.1) were reared in an isolator containing 26 SPF chickens. This served as a control treatment for a concurrent experiment, as facilities were limited at the time.

Table 6.1 Experimental design

Isolator ID	**Chicken type**	**Age at challenge** (days)	**Vaccination status** (Y/N)	**Challenge material**	**n** (number of chickens)
1	Ross	1	Y & N	Buffer	20
2	Ross	1	Y & N	Spleen	20
3	Ross	1	Y & N	Brain	20
4	Ross	1	Y & N	Whole blood	20
5	Ross	1	Y & N	Litter	20
6	Ross	21	Y & N	Buffer	20
7	Ross	21	Y & N	Spleen	20
8	Ross	21	Y & N	Brain	20
9	Ross	21	Y & N	Whole blood	20
10	Ross	21	Y & N	Litter	20
11	SPF	5	N	Spleen	5
12	SPF	5	N	Brain	5
13	SPF	5	N	Whole blood	5
14	SPF	5	N	Litter	5
15	SPF	26	N	Spleen	5
16	SPF	26	N	Brain	5
17	SPF	26	N	Whole blood	5
18	SPF	26	N	Litter	5
19	SPF	5	N	Buffer	5*

*Five SPF chickens run in an isolator which contained a total of 26 SPF chickens

6.2.2 Animals & Management

The experiment used 200 Ross broiler chickens, and 40 SPF (Lohmann LSL classic) chickens (Australian SPF Services Pty Ltd., Woodend, VIC) and was conducted in the UNE poultry isolator facility (Chapter 3). SPF chickens were hatched by the source company and transported by air-freight to UNE for placement into allocated isolators on day -5 of the experiment. Ross broiler chickens were hatched at the hatchery in the production region associated with the APS (Chapter 4) and were transported by air-freight to UNE and placed into isolators on Expt d0.

Ten isolators were utilised for broiler treatment groups and eight isolators were utilised for SPF treatment groups. Twenty chickens were included in each of the broiler isolators and five chickens

in each of the SPF isolators at the commencement of the experiment (Table 6.1). A separate isolator housed 26 SPF chickens concurrently, to serve as sham-treated controls for this experiment and a concurrent experiment using the facility, of which five chickens were identified and used for sampling for this experiment.

Broiler chickens were toe marked on arrival at UNE using a sterile scalpel blade used to make a small cut in the allocated toe web, based on vaccination status as identified by the hatchery. An equal number of both vaccinated and unvaccinated broilers were placed in each allocated isolator.

6.2.3 Treatments

The description of each of the challenge materials utilised in this experiment is provided in Table 6.2 and detailed information regarding the collection and preparation of challenge material used is provided in Chapter 3. Challenge occurred on either Expt d0 or Expt d21. The Expt d0 challenge occurred on the day of hatch for broiler chickens, at which point SPF chickens were 5 days of age. The Expt d21 challenge occurred when broiler chickens were 21 days of age and SPF chickens were 26 days of age. The one sham-challenged SPF chicken group was challenged on day 0 of the experiment.

Table 6.2 Challenge material used

Challenge material	**Detail**
Buffer (sham treatment)	200 µL sterile cell culture medium - Gibco® Medium 199 (Ref: 11150-059) administered via intra-abdominal inoculation
Spleen cells	200 µL ($3.53x10^6$ cells/mL) from affected field birds administered via intra-abdominal inoculation
Brain	200 µL of homogenised tissue from affected field birds administered via intra-abdominal inoculation
Blood	200 µL from affected field birds administered via intra-abdominal inoculation
Litter	100 g from an affected shed in the field added to isolator scratch trays and sprinkled in the feed trough once daily for five consecutive days

6.2.4 Sample collection and chicken maintenance

All broiler isolators had numbers reduced to 12 chickens per isolator (six vaccinated and six unvaccinated chickens where numbers permitted) 14 days post challenge (dpc), thus occurring on Expt d14 for isolators in which chickens were challenged at day-old and Expt d35 for the isolators in which chickens were challenged at 21 days of age. These chickens were humanely euthanased by cervical dislocation and body weight measurements were taken from each chicken. Post-mortem (PM) examinations were then performed, at which time splenic and bursal weights were

recorded and spleens washed with sterile phosphate-buffered saline (PBS) and stored in individually labelled containers at -20°C.

Chickens were inspected twice daily and those demonstrating acute paralysis were humanely euthanased. In most cases euthanasia was performed at the time at which the affected chicken was assessed to be sternally recumbent, unable to walk and could not hold its head and neck off the ground. In fewer cases, euthanasia occurred at the point at which the affected chicken was assessed to be sternally recumbent and unable to walk, but retaining the ability to hold its head and neck off the ground. In just two cases, euthanasia occurred at the point at which the chicken demonstrated an increased tendency to sit, but retained the ability to stand and walk, demonstrating severe ataxia in doing so. Immediately prior to euthanasia, blood samples were collected into both a citrated blood tube (BD Vacutainer® 9NC 0.109M Ref: 363095) and a plain serum tube (BD Vacutainer® Ref: 366668). These chickens were euthanased rapidly via carbon dioxide asphyxiation. Post-mortem (PM) examination was then performed, with brain samples stored both fixed in a 10% neutral buffered formalin solution (4 g sodium phosphate monobasic, 6.5 g sodium phosphate dibasic, 100 mL 37% formaldehyde, 900 mL distilled water) and fresh (-20°C). The process for brain sampling involved initially removing the head from the neck by incising approximately between C2-C3, the mandible was then removed and the skin was removed from the dorsum of the head. The head was then sliced marginally to one side of the midline, with the larger section of the head being placed into the 10% formalin solution. The brain tissue was then removed from the remaining portion of the head with forceps, rinsed with sterile PBS, placed into a sterile container and stored at -20°C.

At the conclusion of the study on Expt d56, at which point broiler chickens were 56 days of age and SPF chickens were 61 days of age, approximately 1 mL of blood was collected from four broilers (two vaccinated and two unvaccinated) from each isolator and all SPF chickens. The blood was collected from a cutaneous ulnar vein and stored in plain serum tubes from which serum was subsequently extracted. All chickens were then euthanased via cervical dislocation and body weight measurements from each bird obtained. PM examinations were performed on each chicken, with bursal and splenic weights recorded, and all spleens being subsequently stored in individually labelled containers at -20°C. Brains from four buffer-inoculated broilers that were inoculated at 21 days of age were collected and fixed as previously described, in order to have control histopathology samples.

The five SPF chickens in the additional isolator housing 26 sham treated control chickens were euthanased when these chickens were 56 days of age, in line with the experimental protocol

associated with a concurrent experiment. Spleen samples were stored from the five identified chickens for this experiment. Blood was collected from each prior to euthanasia, for subsequent serum storage, using the same method as previously described.

6.2.5 Laboratory techniques

6.2.5.1 Histopathology & electron microscopy

Three whole chickens demonstrating acute paralysis were euthanased at UNE and sent immediately chilled to the State Veterinary Diagnostic Laboratory (SVDL) for PM and detailed histological evaluation by avian pathologist Dr. Rod Reece. Fixed brain sections from all remaining chickens that demonstrated acute paralysis and from four apparently normal chickens that were inoculated with buffer at 21 days of age were sent to SVDL for histological and electron microscopy evaluation at the conclusion of the experiment. Sample itemisation for submission was such to blind the pathologist. Initial analysis and reporting was contracted to the SVDL at commercial rates. On completion of reporting, I was able to visit the SVDL and go through all of the sections viewed by Dr. Reece, in order to obtain a greater understanding of the changes and abnormalities present in each.

6.2.5.2 Serology

Serological testing for MDV was performed at UNE utilising the enzyme linked immunosorbent assay (ELISA) technique described in Chapter 3. Serum extracted from whole blood collected from both broiler and SPF chickens at the conclusion of the experiment and stored at -20°C was tested.

Additional serological testing of serum extracted during the experiment was conducted at the Birling Avian Laboratory (Bringelly NSW). Tests at this laboratory utilised ELISA using commercially available kits, except in the cases of Egg Drop Syndrome 1976 (EDS) and ND, both of which were tested for using the technique of haemagglutination inhibition (HI). In the case of EDS, HI was performed using a test developed by SVDL and for ND, using a test developed by the Australian Animal Health Laboratory (AAHL), Victoria. The serological tests and their sources are summarised in Table 6.3.

Table 6.3 Serological tests performed at Birling Avian Laboratories

Disease	Test, Source
Avian encephalomyelitis virus (AEV)	ELISA, IDEXX Laboratories, Batch: 99-09259 JH388, Exp: 9/8/2013
Avian Influenza A (AI)	ELISA, IDEXX Laboratories, Batch: 99-09269 DJ328, Exp: 25/3/2014
Avian leukosis (AL)	ELISA, IDEXX Laboratories, Batch: 99-09257 CJ280, Exp: 25/2/2014
Infectious bursal disease virus (IBDV)	ELISA, IDEXX Laboratories, Batch: 99-09260 LH664, Exp: 11/4/2014
Infectious bronchitis (IB)	ELISA, IDEXX Laboratories, Batch: 99-09262 KH508, Exp: 14/9/2013
Reovirus (Reo)	ELISA, IDEXX Laboratories, Batch: 99-09264 KH495, Exp: 25/10/2013
Reticuloendotheliosis virus (REV)	ELISA, IDEXX Laboratories, Batch: 99-09267 AJ933, Exp: 17/1/2014
Fowl adenovirus serotype 8 (FAV8)	ELISA, TropBio Pty Ltd., Batch: 02-019-01, Exp: Oct 2013
Salmonella typhimurium (ST)	ELISA, x-ovo Ltd., Batch: V020-10413, Exp: Feb 2014
Big liver and spleen disease (BLS)	ELISA, Biocheck Laboratories, Batch: FS5520, Exp: Nov 2013
Egg Drop Syndrome 1976 (EDS)	HI, EMAI, Batch: 29.2
Newcastle disease virus (NDV)	HI, AAHL, Batch: 3-36
Mycoplasma gallisepticum (MG)	ELISA, IDEXX Laboratories, Batch: 99-06729 AJ906, Exp: 19/5/2014
Mycoplasma synoviae (MS)	ELISA, IDEXX Laboratories, Batch: 99-06726 DJ357, Exp: 27/8/2014
Chicken anaemia virus (CAV)	ELISA, IDEXX Laboratories, Batch: 99-08702 KH499, Exp: 18/3/2014

6.2.5.3 Molecular diagnostic techniques

Polymerase chain reaction (PCR) based techniques (Chapter 3) were utilised for testing for the presence of infectious agents in a selection of tissues harvested during the experiment. DNA and RNA were extracted from tissues which had been stored at -20°C from collection (Chapter 3).

PCR for the detection of MDV

DNA amplification reactions were set up (Chapter 3) utilising two different primer sets targeting different areas of the MDV1 genome. DNA extracted from a 42 day old broiler chicken spleen, after vaccination with Rispen's vaccine at day old (Vaxsafe RIS®, Bioproperties Pty Ltd., Batch: RIS8111) was amplified in the same manner for each primer set to verify the effectiveness of the extraction procedure and PCR.

The first set of primers selected were those published by (Silva, 1992), using oligonucleotide sequences flanking the 132-base-pair (bp) tandem direct repeats (TDR's) in the MDV1 genome. The forward primer was named pp38SilvaFWD and the reverse primer identified as pp38SilvaREV, their sequences are listed in Table 6.4. Amplification reactions run on gels show bands that are 132 bp and larger, in increments of 132 bp depending on the number of TDR's.

The second set of primers selected were 18 nucleotides long (Table 6.4), complementary to the *meq* gene of the MDV1 genome, flanking a 577 bp region, as used by Wajid *et al.* (2013). The 5' end of the forward primer (BCH342) is 21 bp upstream from the start codon of the *meq* gene and the reverse primer (SJW1) is within the *meq* gene.

Table 6.4 Sequence of primers used in PCR tests for Marek's disease virus, avian encephalomyelitis virus and flaviviruses

Identification	**Virus**	**Target gene**	**Sequence**	**Reference**
pp38Silva FWD	MDV1	132 bp TDR's	5' TGCGATGAAAGTGCTATGGAGG 3'	Silva, 1992
pp38Silva REV	MDV1	132 bp TDR's	5' GAGAATCCCTATGAGAAAGCGC 3'	Silva, 1992
BCH342	MDV1	*meq*	5' ATTCCGCACACTGATTCC 3'	Wajid et al., 2013
SJW1	MDV1	*meq*	5' AGCAATGTGGAGCGTTAG 3'	Wajid et al., 2013
MKAE1FWD	AEV	*VP2*	5' CTTATGCTGGCCCTGATCGT 3'	Xie et al., 2005
MKAE2REV	AEV	*VP2*	5' TCCCAAATCCACAAACCTAGCC 3'	Xie et al., 2005
FU2	FLA	*NS5*	5' GCTGATGACACCGCCGGCTGGGACAC 3'	Kuno et al., 1998
cFD3	FLA	*NS5*	5' AGCATGTCTTCCGTGGTCATCCA 3'	Kuno et al., 1998

Aliquots of the amplification reactions were analysed on agarose mini-gels (Chapter 3) that contained 2% and 0.8% agarose respectively for the pp38 and *meq* amplification products.

Real-time PCR (qPCR)

Quantitative PCR (qPCR) was used to test for the presence of MDV1 and HVT in a selection of tissues harvested from the experiment and the assay is described in detail in Chapter 3.

Reverse-transcriptase PCR (RT-PCR) for the detection of AEV and flaviviruses

The oligonucleotide primers developed by Xie *et al.* (2005) were used to amplify AEV. The primers MKAE1FWD and MAKE2REV (Table 6.4) flank a 619 bp DNA sequence containing the *VP2* gene in AEV. The oligonucleotide pan-flavivirus primers (Table 6.4) reported by Kuno *et al.* (1998), amplify an 845 nucleotide sequence of the viral gene encoding non-structural protein 5 (NS5) of all flaviviruses and were used in an RT-PCR (Chapter 3). RNA extracted from AEV vaccine (Poulvac® AE I, Zoetis Australia, Batch: 1573128A) and from Palm Creek virus (provided by the Australian Infectious Diseases Research Centre, University of Queensland) were put through the same RT-PCR to verify the efficacy of the AEV and pan-flavivirus RT-PCR's respectively. Aliquots of the amplification reactions were analysed on agarose mini-gels (Chapter 3) that contained 1% and 0.8% agarose respectively for the AEV and pan-flavivirus amplification products.

6.2.6 Statistical Analysis

Statistical analysis of experimental data was conducted using the software package JMP 10 (SAS Institute Inc., 2012). A significance level of $P < 0.05$ was accepted for all test statistics. Relative immune organ weights (bursa of Fabricius and spleen) were expressed as a percentage of bodyweight (relative organ weight = [(organ weight/bodyweight) x 100]).

The chi-square test statistic was used in analysing proportions in contingency tables, except in cases when the assumptions relating to expected frequencies could not be met (values below 5), in which case Fisher's exact test was used. Time to event analyses, quantifying the time taken for birds to die or require euthanasia (due to the APS) following enrolment into the study (in days), were described using Kaplan-Meier survival curves.

For continuous variables including bodyweight and relative immune organ weights, distributions were assessed for normality and distribution of residuals to assess the need for transformation, from which none was required. Analyses were conducted using a general linear model, fitting the effects of sex, vaccination status, challenge material, age at challenge and PM operator from data recorded on Expt d56 and for sex, challenge material and PM operator on smaller sets of data recorded on Expt d14 and Expt d35. From such data, least-square means and standard errors are presented, and Tukey's HSD test was used to determine the significance of differences between more than two means.

Analysis of discrete serological data (positive or negative) was performed using a general linear model with a binomial link function (logistic) fitted for the effects of challenge and age at challenge.

6.3 Results

6.3.1 Reproduction of the APS

Reproduction of the APS was successful in a number of Ross broiler chickens in treatment groups that were inoculated with spleen or blood at 21 days of age or exposed to litter at 21 days of age (Table 6.5). The syndrome was not observed in similar groups treated at day-old, in any group inoculated with brain or buffer, or in any SPF chicken. Affected broilers were only confirmed to have developed the APS if the clinical signs as described in Table 6.6 were observed.

Table 6.5 Summary of chickens which developed the APS

Bird serial ID	Challenge material	Age at challenge (days)	HVT vacc. status (Y/N)	Sex	Age at onset (days)	Age at euth. (days)	Duration of clinical signs (days)*
54	Blood	21	No	F	30	33	3
55	Blood	21	No	M	31	33	2
56	Blood	21	No	M	31	33	2
57	Blood	21	Yes	F	31	33	2
58	Blood	21	No	M	31	33	2
59	Spleen	21	No	M	29	33	4
60	Spleen	21	No	M	31	33	2
87	Spleen	21	Yes	F	37	40	3
89	Litter	21	Yes	F	37	40	3
93	Blood	21	Yes	F	44	47	3
94	Blood	21	No	M	45	47	2
95	Spleen	21	Yes	M	43	47	4
96	Spleen	21	Yes	M	45	47	2
105	Spleen	21	No	F	48	50	2
109	Litter	21	Yes	F	48	50	2
110	Spleen	21	No	F	49	51	2

*Time from first observation of clinical signs until euthanasia

The typical progression of the APS in affected chickens was initially an increased tendency to sit and a reluctance to stand and walk when prompted. At this point when walking, chickens demonstrated a paretic and wide-based gait. Over a period of 1-3 days this progressed to an obtunded state with chickens being completely unable to stand and walk on prompting and a tendency for the head and neck to be resting on the floor of the isolator; however, both could be held upright if the chicken was prompted. At the final stage of the condition affected chickens were stuporous, demonstrated a complete inability to hold their head and neck off the floor and marked flaccidity of the neck. The third stage was typically reached within four days of the onset of the first stage. Affected chickens retained the ability to eat and drink if feed and water was placed directly in front of them, up until the third stage. At all stages affected chickens retained vision (positive menace response), eye-ball position remained normal bilaterally, retained beak tone and tongue movement, demonstrated the presence of a palpebral response, did not regurgitate and responded to painful stimuli (pinch) to the feet. Additionally affected chickens demonstrated normal vent-sphincter, pedal-flexor and wing withdrawal reflexes. At stages when chickens were completely recumbent and unable to stand, they retained proprioception as demonstrated by their ability to return wings to a folded position, to return legs and feet beneath their body if moved and to return themselves to sternal recumbency if they were pushed from this position.

Based on the progression of clinical signs observed, the syndrome could be summarised into three sequential stages (Table 6.6) and photographed examples are provided for each in Figure 6.1. Due

to university animal ethics guidelines, no chicken was allowed to progress to death in the induced paralytic state. Most chickens were euthanased when they were in stage 3 paralysis; however, in a few cases euthanasia was performed at stages 1 or 2. This is outlined for individual chickens in Table 6.9. Recovery from any affected stage was not observed in any case. All affected chickens progressed through each sequential stage, excluding the cases whereby euthanasia occurred prior to stage 3.

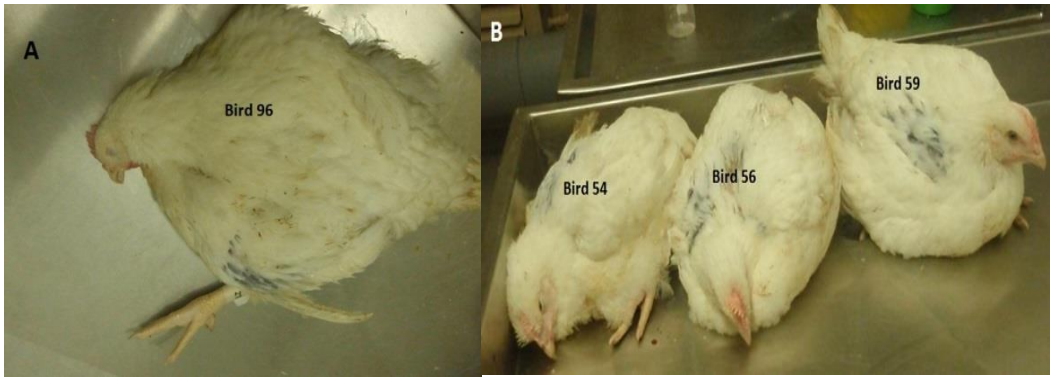

Figure 6.1 Photographs of experimental chickens demonstrating acute paralysis. A: Bird serial number 96, demonstrating stage 3 of the APS immediately prior to euthanasia. B: Bird serial numbers 54, 56 and 59 demonstrating stages 3, 2, and 1 of the APS respectively, immediately prior to euthanasia.

Table 6.6 Summary of the clinical signs observed at each sequential stage of the APS as allocated in this experiment

Stage 1	• Increased tendency to sit • Reluctance to stand and walk • Paretic, ataxic and wide-based gait • Ability to eat and drink • Postural reactions normal • Cranial nerves functioning • Spinal reflexes intact • Nociception intact
Stage 2	• Obtundation • Complete inability to stand or walk • Head and neck lie on floor most of the time, however can be lifted and held up if bird was prompted • Postural reactions normal • Cranial nerves functioning • Spinal reflexes intact • Nociception intact
Stage 3	• Stuporous • Complete inability to stand or walk • Head and neck lie on floor permanently and could not be lifted and held up if bird was prompted • Unable to eat and drink even if such was provided directly in front of the bird • Postural reactions normal • Cranial nerves functioning • Spinal reflexes intact • Nociception intact.

In considering the effect of sex on the likelihood of a broiler developing the APS, males were more susceptible to the syndrome then were females (P = 0.04), with 8/55 males and 8/145 females developing the APS. HVT vaccination did not provide a protective effect against the development of the APS in broiler chickens (P = 0.24), with 7/100 vaccinated and 9/100 unvaccinated chickens developing the APS.

6.3.2 Mortality & Survivability

Total mortality and that due to the APS for each experimental group is provided in Table 6.7. Survivability was analysed by the variety of chicken and age at challenge, via the Kaplan-Meier approach. Chickens euthanased at 14 days post challenge for routine diagnostics were excluded from all survival analyses. Additionally, several chickens that died of accidental causes, such as death due to handling at challenge were also excluded from these analyses. Other deaths from various causes unrelated to the APS throughout the experiment were included in such analyses, on the assumption of these being evenly spread through both sexes. Total mortality was analysed given that biological material was used for challenge which may have contained any of a range of infectious agents.

Survival patterns of broilers are shown in Figure 6.2 and for SPF chickens in Figure 6.3. There was a significant effect (P < 0.005) of challenge material on broiler survival when challenged on Expt d21 (Figure 6.2.B). The effect of challenge material on survival of broilers challenged on Expt d0 (P = 0.13) (Figure 6.2.A) and in SPF chickens challenged on either Expt d0 (P = 0.11) (Figure 6.3.A) or Expt d21 (P = 0.36) (Figure 6.4.B) were non-significant, which is consistent with the APS not being observed in these groups.

Table 6.7 Summary of mortality due to the APS or other causes by experimental factor

Breed	Challenge material	Expt day of challenge	Vaccination status	Number placed	Total mortality	Mortality due to APS
Ross	Buffer	1	Vaccinated	10	0/10	-
Ross	Buffer	1	Unvaccinated	10	0/10	-
Ross	Buffer	21	Vaccinated	10	0/10	-
Ross	Buffer	21	Unvaccinated	10	0/10	-
Ross	Spleen	1	Vaccinated	10	1/10	0/1
Ross	Spleen	1	Unvaccinated	10	0/10	-
Ross	Spleen	21	Vaccinated	10	**4/10**	**3/4**
Ross	Spleen	21	Unvaccinated	10	**5/10**	**4/5**
Ross	Blood	1	Vaccinated	10	2/10	0/2
Ross	Blood	1	Unvaccinated	10	2/10	0/2
Ross	Blood	21	Vaccinated	10	**5/10**	**2/5**
Ross	Blood	21	Unvaccinated	10	**6/10**	**5/6**
Ross	Brain	1	Vaccinated	10	0/10	-
Ross	Brain	1	Unvaccinated	10	1/10	0/1
Ross	Brain	21	Vaccinated	10	4/10	0/4
Ross	Brain	21	Unvaccinated	10	3/10	0/3
Ross	Litter	1	Vaccinated	10	1/10	0/1
Ross	Litter	1	Unvaccinated	10	1/10	0/1
Ross	Litter	21	Vaccinated	10	**5/10**	**2/5**
Ross	Litter	21	Unvaccinated	10	1/10	0/1
SPF	Buffer	1	Unvaccinated	5	0/5	-
SPF	Spleen	1	Unvaccinated	5	2/5	0/2
SPF	Spleen	21	Unvaccinated	5	0/5	-
SPF	Brain	1	Unvaccinated	5	2/5	0/2
SPF	Brain	21	Unvaccinated	5	1/5	0/1
SPF	Blood	1	Unvaccinated	5	0/5	-
SPF	Blood	21	Unvaccinated	5	0/5	-
SPF	Litter	1	Unvaccinated	5	0/5	-
SPF	Litter	21	Unvaccinated	5	0/5	-

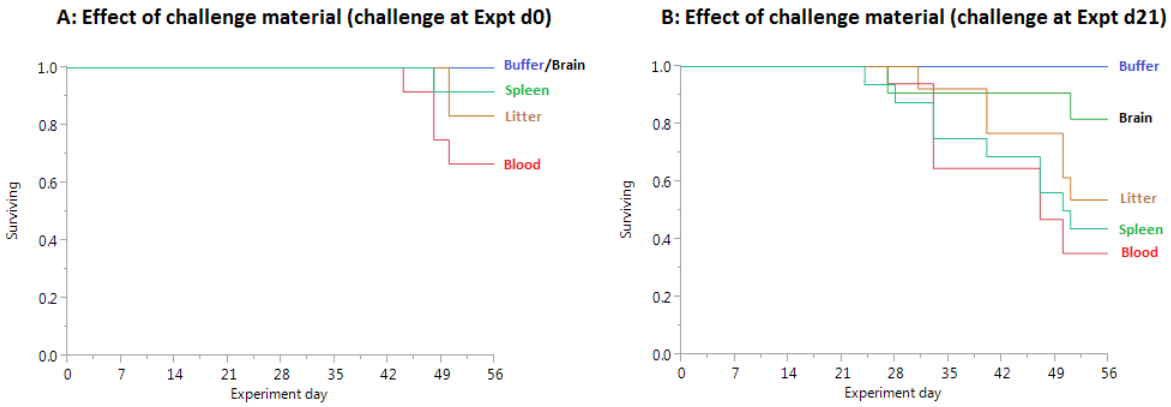

Figure 6.2 Survival patterns of broiler chickens challenged on Expt d0 (P = 0.13) (A) and Expt d21 (P < 0.005) (B) by challenge material grouping.

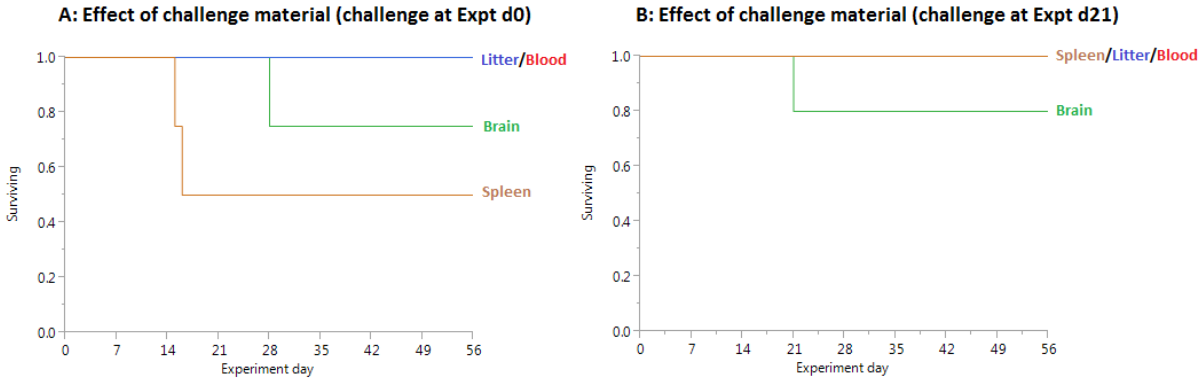

Figure 6.3 Survival patterns of SPF chickens challenged on Expt d0 (P = 0.11) (A) and Expt d21 (P = 0.36) (B) by challenge material grouping.

Similar survival analyses were conducted considering any effect associated with sex and HVT vaccination status. A significant effect of sex was found in broilers challenged on Expt d21, with significantly reduced survivability of males as opposed to females (P = 0.02) (Figure 6.4.A). The effects of sex on survival of broilers challenged on Expt d0 and SPF chickens challenged on Expt d0 and Expt d21 were non-significant. The effect of HVT vaccination on survivability was non-significant for all groups (Figure 6.4.B).

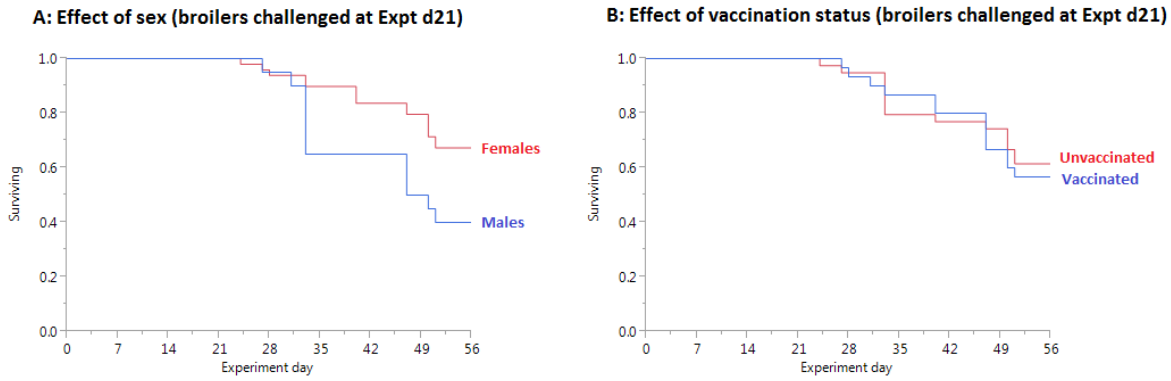

Figure 6.4 Survival patterns of Ross broiler chickens challenged at 21 days of age. A: Effect of sex on survival post-challenge. The differences between the sexes is significant (P = 0.02). B: Effect of HVT vaccination on survival post-challenge did not influence survival (P = 0.69).

6.3.3 Bodyweights and relative immune organ weights

Table 6.8 summarises analyses conducted on bodyweight and immune organ weight data recorded on Expt d14 (chickens challenged Expt d0), Expt d35 (chickens challenged Expt d21) and Expt d56. Sham-challenged SPF chickens (Table 6.1) were excluded from these analyses as they were run at a significantly higher stocking density than all other SPF groups.

Table 6.8 Analysis of bodyweight (BWt) and relative bursal and spleen weight data, presenting least squares means ± standard error and P-values showing the effect of each experimental factor/s

Parameter/Level/Effect	BWt (g)	Rel. bursal wt (%)	Rel. spleen wt (%)
Broilers - Experiment day 14	492±8	0.17±0.01	0.08±0.003
SEX	**P = 0.02**	P = 0.11	P = 0.34
Male	$528±17^{A}$	0.1488±0.0142	0.0877±0.0068
Female	$481±9^{B}$	0.1750±0.0077	0.0802±0.0037
CHALLENGE MATERIAL	**P = 0.03**	P = 0.27	P = 0.1
Buffer	$542±18^{A}$	0.199±0.015	0.0715±0.0069
Spleen	$519±24^{AB}$	0.166±0.015	0.0723±0.0069
Brain	$491±18^{AB}$	0.153±0.015	0.0934±0.0069
Blood	$514±16^{AB}$	0.161±0.015	0.0830±0.0069
Litter	$441±24^{B}$	0.167±0.015	0.0894±0.0069
POST-MORTEM OPERATOR	n/a	P = 0.06	P = 0.51
SEX * CHALLENGE MATERIAL	P = 0.26	P = 0.41	P = 0.81
Broilers - Experiment day 35	1969±56	0.12±0.01	0.10±0.01
SEX	P = 0.15	P = 0.44	P = 0.25
Males	2045±79	0.1098±0.0113	0.0884±0.0106
Females	1896±63	0.1211±0.0089	0.1044±0.0083
CHALLENGE MATERIAL	P = 0.77	P = 0.22	P = 0.05
Buffer	1944±90	0.094±0.012	0.072±0.010
Spleen	2156±143	0.117±0.017	0.113±0.015
Brain	1863±143	0.118±0.017	0.126±0.015
Blood	2210±152	0.140±0.020	0.089±0.017
Litter	1952±104	0.132±0.013	0.107±0.011
POST-MORTEM OPERATOR	n/a	P = 0.32	P = 0.78
SEX * CHALLENGE MATERIAL	P = 0.25	P = 0.48	P = 0.69
Broilers - Experiment day 56	3450±56	0.09±0.004	0.105±0.003
SEX	**P < 0.0001**	**P = 0.02**	**P = 0.04**
Males	$3886±76^{A}$	$0.1057±0.0077^{A}$	$0.1207±0.0085^{A}$
Females	$3338±39^{B}$	$0.0859±0.0039^{B}$	$0.1008±0.0043^{B}$
CHALLENGE MATERIAL	P = 0.96	P = 0.66	**P = 0.004**
Buffer	3531±115	0.0818±0.0093	$0.078±0.010^{B}$
Spleen	3542±120	0.0906±0.0097	$0.105±0.010^{AB}$
Brain	3507±120	0.0745±0.0097	$0.104±0.010^{AB}$
Blood	3526±141	0.0960±0.0114	$0.138±0.012^{A}$
Litter	3418±126	0.0875±0.0102	$0.088±0.011^{B}$
AGE AT CHALLENGE	P = 0.29	P = 0.11	P = 0.19
Day-old	3505±56	0.0861±0.0045	0.1024±0.0049
21 days	3413±66	0.0970±0.0052	0.1124±0.0058
VACCINATION STATUS	P = 0.51	P = 0.44	P = 0.38
Vaccinated	3476±71	0.0891±0.0057	0.0991±0.0061
Unvaccinated	3534±71	0.0836±0.0057	0.1057±0.0062
POST-MORTEM OPERATOR	n/a	P = 0.65	P = 0.19
SEX * CHALLENGE MATERIAL	P = 0.41	P = 0.18	P = 0.50

Parameter/Level/Effect	BWt (g)	Rel. bursal wt (%)	Rel. spleen wt (%)
SEX * AGE AT CHALLENGE	P = 0.09	P = 0.14	P = 0.82
SEX * VACCINATION STATUS	P = 0.19	P = 0.54	P = 0.89
CHALLENGE MATERIAL * AGE AT CHALLENGE	P = 0.45	P = 0.09	P = 0.68
CHALLENGE MATERIAL * VACCINATION STATUS	P = 0.18	P = 0.38	P = 0.16
AGE AT CHALLENGE*VACCINATION STATUS	P = 0.63	P = 0.07	P = 0.58
SPF chickens - Experiment day 56	894±19	0.252±0.022	0.251±0.008
SEX	**P < 0.0001**	P = 0.24	**P= 0.01**
Males	$956±15^{A}$	0.232±0.027	$0.236±0.010^{B}$
Females	$777±21^{B}$	0.288±0.038	$0.280±0.013^{A}$
CHALLENGE MATERIAL	P = 0.14	**P = 0.001**	**P = 0.001**
Spleen	759±62	$0.174±0.035^{B}$	$0.239±0.018^{AB}$
Brain	959±62	$0.346±0.035^{A}$	$0.230±0.018^{B}$
Blood	853±48	$0.372±0.027^{A}$	$0.300±0.014^{A}$
Litter	899±48	$0.297±0.027^{A}$	$0.209±0.014^{B}$
AGE AT CHALLENGE	P = 0.20	**P < 0.001**	P = 0.18
Day-old	867±28	$0.297±0.016^{A}$	0.2431±0.0079
21 days	916±25	$0.212±0.014^{B}$	0.2579±0.0071
POST-MORTEM OPERATOR	n/a	n/a	n/a
SEX * CHALLENGE MATERIAL	P = 0.14	P = 0.63	P = 0.14
SEX * AGE AT CHALLENGE	P = 0.17	P = 0.92	P = 0.17
CHALLENGE MATERIAL * AGE AT CHALLENGE	P = 0.67	**P = 0.001**	**P = 0.03**
Spleen/day-old	759±62	$0.17±0.04^{BC}$	$0.239±0.018^{ABC}$
Spleen/21 days	873±48	$0.12±0.03^{C}$	$0.265±0.014^{AB}$
Brain/day-old	959±62	$0.35±0.04^{A}$	$0.230±0.018^{ABC}$
Brain/21 days	939±53	$0.33±0.03^{AB}$	$0.294±0.015^{A}$
Blood/day-old	853±48	$0.37±0.03^{A}$	$0.295±0.014^{A}$
Blood/21 days	885±48	$0.35±0.03^{A}$	$0.290±0.014^{A}$
Litter/day-old	899±48	$0.30±0.03^{AB}$	$0.209±0.014^{BC}$
Litter/21 days	969±48	$0.05±0.03^{C}$	$0.182±0.014^{C}$

^{A,B,C} Figures with a different superscript differ significantly, specific to each level only.

6.3.3.1 Bodyweights

For broilers challenged on Expt d0, sex had significant effect on body weight, with males being 10% heavier than females (Table 6.8). Additionally, challenge material had a significant effect on bodyweight at this point due to litter challenged chickens being 19% lighter than buffer challenged chickens (Figure 6.5). The effect of the interaction between sex and challenge material was non-significant in these chickens (Table 6.8).

For broilers challenged on Expt d21, the effects on bodyweight of sex, challenge material, and the interaction between sex and challenge material were non-significant (Table 6.8). However, given the experiment was designed to reduced bird numbers in each broiler isolator to 12 birds at this point and with large number of mortalities recorded just prior to this day mostly due to the APS, relatively fewer birds were sampled on Expt d35 as compared to Expt d14.

On Expt d56 male broilers were 16% heavier than females (Table 6.8). At this point, the effects on bodyweight of challenge material, age at challenge, vaccination status and the interactions between sex and challenge material, sex and age at challenge, sex and vaccination status, challenge material and age at challenge, challenge material and vaccination status and age at challenge and vaccination status were all non-significant (Table 6.8). On Expt d56 male SPF chickens were 23% heavier than females (Table 6.8). The effects on bodyweight of challenge material, age at challenge and the interactions between sex and challenge material, sex and age at challenge, and challenge material and age at challenge were all non-significant (Table 6.8).

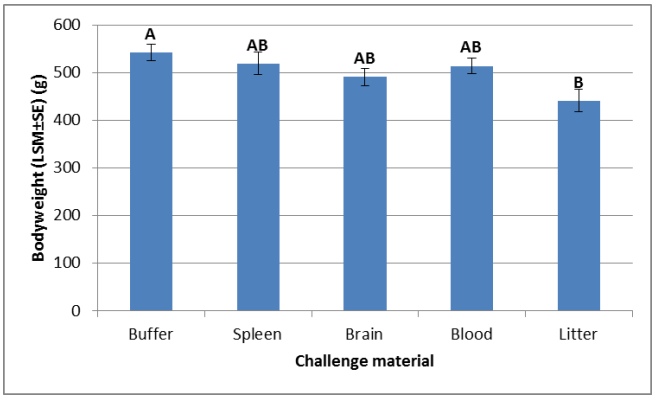

Figure 6.5 Least squares means (±SE) for bodyweights by challenge material in broilers on Expt d0 (columns not sharing a common letter differ significantly).

6.3.3.2 Bursa of Fabricius

For broilers on Expt d14 and Expt d35, the effect on relative bursal weight from sex, challenge material and PM operator were non-significant (Table 6.8). On Expt d56 males had 23% greater relative bursal weight than females (Table 6.8). On Expt d56, the effects on relative bursal weight from challenge material, age at challenge, vaccination status, PM operator and the interactions between sex and challenge material, sex and age at challenge, sex and vaccination status, challenge material and age at challenge, challenge material and vaccination status, and age at challenge and vaccination status were all non-significant (Table 6.8).

For SPF chickens on Expt d56, challenge material, age at challenge and the interaction between challenge material and age at challenge all had significant effects on relative bursal weight due to significant bursal atrophy in litter and spleen challenged chickens and significant variance in such between chickens challenged with litter on Expt d0 compared to those challenged on Expt d21

(Table 6.8, Figure 6.6). In these chickens the effect on relative bursal weight from sex and the interactions between of sex and challenge material and sex and age at challenge were all non-significant (Table 6.8).

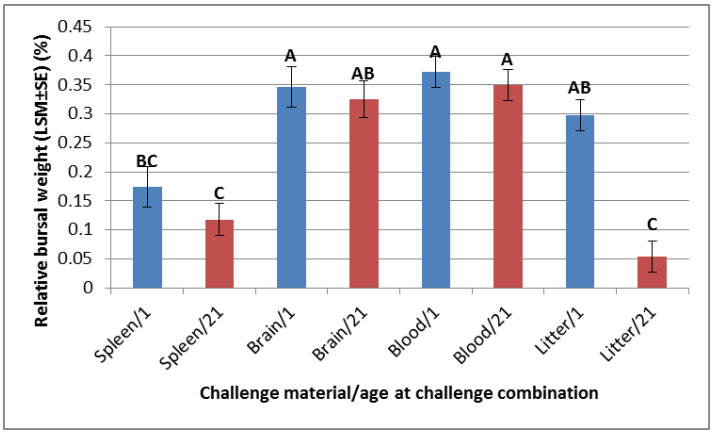

Figure 6.6 Least squares means (±SE) for relative bursal weights on Expt d56 by treatment and age at treatment (days) for SPF chickens (columns not sharing a common letter differ significantly).

6.3.3.3 Spleen

For broilers on Expt d0 and Expt d21, the effect on relative spleen weight from sex, challenge material, PM operator and the interaction between sex and challenge material were all non-significant (Table 6.8). On Expt d56 male broilers had 20% greater relative spleen weight than females (Table 6.8). Challenge material had a significant effect on relative spleen weight due to mean relative spleen weights of litter and buffer challenged chickens being significantly less than that of blood challenged chickens (Table 6.8, Figure 6.7.A). The effect on relative spleen weight of age at challenge, vaccination status, PM operator and the interactions between sex and challenge material, sex and age at challenge, sex and vaccination status, challenge material and age at challenge, challenge material and vaccination status and age at challenge and vaccination status were all non-significant (Table 6.8).

On Expt d56 female SPF chickens had 19% greater relative spleen weight than males (Table 6.8). Challenge material and the interaction between challenge material and age at challenge had significant effects on relative spleen weight due to the mean relative spleen weights for blood challenged chickens being significantly greater than that for brain and litter challenged chickens (Table 6.8, Figure 6.7.B). The effect on SPF relative spleen weight from age at challenge and the interaction between sex and challenge material and sex and age at challenge were all non-significant (Table 6.8).

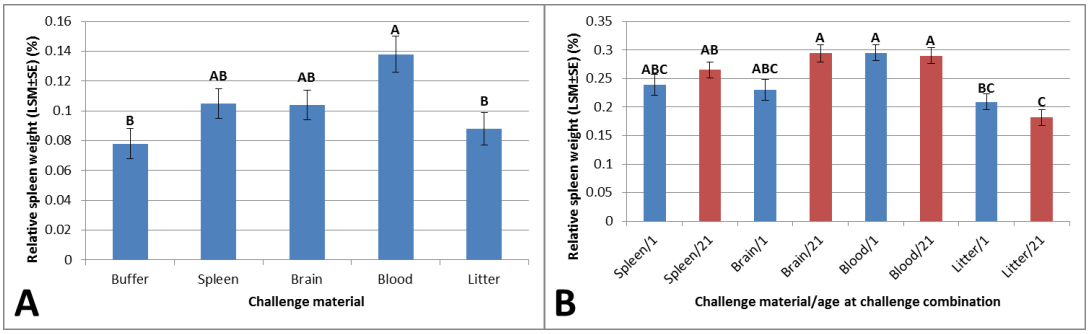

Figure 6.7 Least squares means (±SE) relative spleen weights. A: Broilers on Expt d56 by challenge material. B: SPF chickens on Expt day 56 by challenge material and age at challenge groupings. Columns not sharing a common letter differ significantly.

6.3.4 Histopathology and electron microscopy

Table 6.9 provides a summary of clinical and histopathological findings of individual chickens that demonstrated the APS, identifying the challenge material, age at challenge, sex, vaccination status, age at euthanasia, clinical signs and histopathological lesions observed in the brain. Histological evaluation of the spinal cord was performed only for birds 54, 56 and 59 which were euthanized, immediately chilled, and sent to the SVDL. Birds 54, 56 and 59 demonstrated histopathology in the brain and spinal cord. All other organ systems which were evaluated grossly and histologically in these birds were considered normal, including: peripheral nerves, thymus, bursa of Fabricius, ventral feather tract, kidney, adrenal gland, spleen, lung, trachea, thyroid and parathyroid gland. The histopathology observed in the fixed brains submitted at the conclusion of the trial were consistent with those changes observed in the three birds submitted earlier. The most consistent histopathological lesion observed in fixed brain sections was perivascular cuffing of mononuclear cells in the cerebrum. This change was invariably observed with progression to vasculitis, characterised by presence of perivascular oedema (Figure 6.8 A-D) and hypertrophy of vascular endothelium (Figure 6.8 B,D). In some sections, infiltration of mononuclear cells into adjacent neuropil was observed. Pathology was not detected in brain sections from sham-challenged chickens (Table 6.9).

Similar vascular pathology was noted in spinal cords of birds 54, 56 and 59 with the presence of perivascular cuffing and occasional perivascular oedema and endothelial hypertrophy. In addition to these changes marked degenerative changes were found in the two spinal cords, at variable locations. Wallerian degeneration was found in these two cords with the presence of swollen nerve axons, strings of oligodendroglial cells, the presence of occasional degenerate macrophages and the presence of numerous digestion chambers (Figure 6.8.F). The presence of glial nodules was not a consistent finding but they were observed focally in a small number of brains and spinal cords from affected chickens.

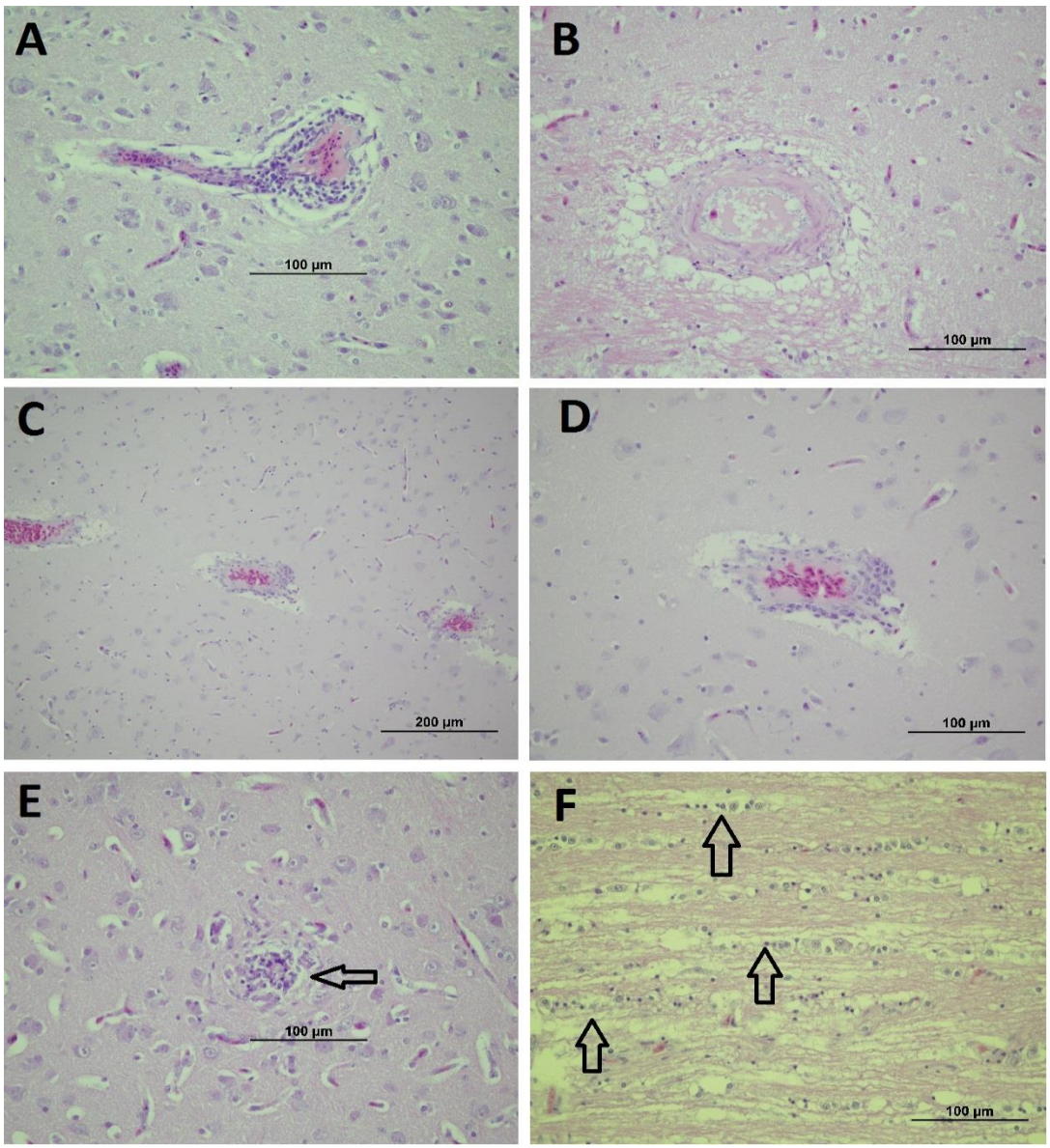

Figure 6.8 A: vessel in the base of the cerebrum demonstrating perivascular cuffing of mononuclear cells and associated perivascular oedema; B: vessel in cerebrum demonstrating endothelial hypertrophy and perivascular oedema giving a 'scalloped' appearance, there is a mild mononuclear infiltrate; C: sections of vessels in cerebrum with perivascular cuffing of mononuclear cells and some associated perivascular oedema; D: a closer view of the central vessel in C, demonstrating endothelial hypertrophy; E: a vessel (arrow) in the cerebrum demonstrating perivascular cuffing of mononuclear cells, highlighting how subtle some of these lesions were; F: section of spinal cord demonstrating wallerian degeneration, including swollen axons and the presence of digestion chambers containing cell debris (arrows). Photos by Dr. Rod Reece, SVDL.

Table 6.9 Summary of clinical and histopathological findings of individual chickens demonstrating the APS (histopathological interpretations provided by Dr. Rod Reece, SVDL)

Bird serial ID	Challenge and age at challenge	Sex	Age (days)	HVT vacc. status (Y/N)	Clinical signs	Histopathological lesions
54	Blood d21	F	33	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** perivascular cuffing of mononuclear cells around several vessels in caudal cerebrum. **Spinal Cord (lumbar):** diffuse wallerian degeneration, marked vacuolation in the longitudinal tracts, presence of chains of digestion chambers and strings of oligodendroglial cells, the presence of an occasional degenerate macrophage in vacuolar space.
55	Blood d21	M	33	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** several veins exhibiting a narrow mononuclear cell rim, associated endothelium normal.
56	Blood d21	M	33	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck held on floor but can be maintained in normal position for a small period of time if prompted. **Stage 2.**	**Brain:** perivascular cuffing of mononuclear cells around several vessels in caudal cerebrum, associated with mild endothelial hypertrophy. **Spinal Cord (cervical):** perivascular cuffing of mononuclear cells associated with meninges, heterophils present in vessel walls and adjacent tissue. **Spinal Cord (thoracic):** perivascular cuffing of mononuclear cells associated with the meninges and ganglia. **Spinal Cord (lumbar):** no significant findings.
57	Blood d21	F	33	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck held on floor but can be maintained in normal position for a small period of time if prompted. **Stage 2.**	**Brain:** one vein deep in the cerebrum has a thick, dense cuff of mononuclear cells.
58	Blood d21	M	33	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck held on floor but can be maintained in normal position for a small period of time if prompted. **Stage 2.**	**Brain:** one arteriole deep in the cerebrum has a dense mononuclear cell infiltrate, with associated marked endothelial hypertrophy. A group of arterioles close to the associated ventricle show mononuclear perivascular cuffing and perivascular oedema.

59	Spleen d21	M	33	No	Sternal recumbency which can be maintained, able to stand and walk but demonstrates severe ataxia, head and neck held in a normal position. **Stage 1.**	**Brain:** several small vessels deep in the cerebrum demonstrating mononuclear perivascular cuffing and associated perivascular oedema. Two larger vessels at the base of the cerebrum demonstrating wider perivascular cuffing of mononuclear cells. **Spinal Cord (cervical):** moderate perivascular infiltrate of mononuclear cells associated with multiple meningeal veins, one small focus of gliosis associated with a meningeal vessel. **Spinal Cord (thoracic):** multiple meningeal vessels demonstrating narrow mononuclear perivascular cuffing, associated perivascular oedema prominent. **Spinal Cord (lumbar):** wallerian degeneration prominent, vacuolation also prominent, multiple vessels in ganglia and gray matter demonstrating mononuclear perivascular cuffing and associated oedema.
60	Spleen d21	M	33	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck tending to be held low but can be maintained in a normal position when bird prompted. **Stage 2.**	**Brain:** multiple arterioles and veins deep in cerebrum display endothelial hypertrophy and thick perivascular cuffing of mononuclear cells.
87	Spleen d21	F	40	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** one small vessel at base of the cerebrum demonstrating mild-moderate mononuclear cell perivascular cuffing and infiltration, with similar observations for one vein in the optic tectum.
89	Litter d21	F	40	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** one vein deep in the cerebrum has a focal accumulation of mononuclear cells which is not completely circumferential, significant perivascular oedema associated with this vessel.
93	Blood d21	F	47	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** a vein deep in the cerebrum with obvious perivascular cuffing of mononuclear cells, and associated perivascular oedema.
94	Blood d21	M	47	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** several vessels deep in the cerebrum with perivascular cuffing of mononuclear cells and associated perivascular oedema.
95	Spleen d21	M	47	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** several vessels deep in the cerebrum with perivascular cuffing of mononuclear cells and associated perivascular oedema, moderate amount of vacuolation throughout cerebrum.

96	Spleen d21	M	47	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** several vessels at the base of the cerebrum demonstrating thin mononuclear perivascular cuffs, with a focal glial cell granuloma adjacent to one of the affected vessels.
105	Spleen d21	F	50	No	Sternal recumbency which can be maintained, able to stand and walk but demonstrates severe ataxia, head and neck held in a normal position. **Stage 1.**	**Brain:** several vessels at the base of the cerebrum demonstrating thin mononuclear perivascular cuffs.
109	Litter d21	F	50	Yes	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** several vessels at the base of the cerebrum demonstrating mononuclear perivascular cuffs and endothelial hypertrophy.
110	Spleen d21	F	51	No	Sternal recumbency which can be maintained, unable to stand or walk, head and neck complete flaccid paralysis. **Stage 3.**	**Brain:** multiple small vessels in the base of the cerebrum and within the cerebellum demonstrating mononuclear perivascular cuffing, endothelial hypertrophy and perivascular oedema.
113	Buffer d21	F	56	No	Abnormal clinical signs not observed.	**Brain:** no visible lesions.
114	Buffer d21	F	56	Yes	Abnormal clinical signs not observed.	**Brain:** no visible lesions.
115	Buffer d21	F	56	Yes	Abnormal clinical signs not observed.	**Brain:** no visible lesions.
116	Buffer d21	F	56	No	Abnormal clinical signs not observed.	**Brain:** no visible lesions.

Several brain sections with marked inflammatory pathology at the base of the cerebrum such as those shown in Figures 6.8 A, C and D were selected for evaluation by electron microscopy in order to detect any associated viral particles or other abnormalities. The process required the retrieval of the corresponding blocks of fixed tissue so that fresh sections could be cut and processed suitably for evaluation by transmission electron microscopy. No viral particles or other abnormalities were detected in any section.

6.3.5 Serology

6.3.5.1 Marek's Disease

A concentration titre of 569 units was selected as the cut-off value for the MDV ELISA, with values > 569 being defined as MDV seropositive. The selected titre was the highest titre derived from the five sham-challenged SPF chicken sera tested. The MDV ELISA on serum from eight sham-challenged broilers was consistent with effective HVT vaccination. All unvaccinated broilers were MDV negative, with mean titre (±SE) 464±168 and all vaccinated broilers were MDV positive with mean titre (±SE) of 1056±168.

Serology for MDV was performed on samples from 40% of the SPF chicken population collected on Expt d56, representing all treatment groups except those inoculated with brain. Samples tested from sham-challenged SPF chickens had mean titre (±SE) 465±38. All samples tested from litter challenged groups were MDV negative with a mean titre (±SE) 111±111. All samples tested from blood and spleen challenged SPF chickens were MDV positive with mean titres (±SE) 848±104 and 4219±304 respectively. These results suggest that MDV was present in both of the blood and spleen inocula and was subsequently transmitted to experimental chickens as a result of inoculation.

All broilers which succumbed to the APS were found to be MDV positive (Table 6.10) with mean titre (±SE) 4497±1034. Both litter-challenged broiler chickens developing the APS had been HVT vaccinated so the positive values were likely associated with either horizontal transfer from vaccinated chickens or vaccination, given that litter-challenged SPF chickens were MDV seronegative.

Table 6.10 Serological results from a portion of individual chickens that developed the APS

Bird ID	Challenge	Age at challenge (d)	Age at euth.(d)	HVT Vacc. (Y/N)	AEV	AI	AL	IBDV	IB	Reo	REV	FAV8	ST	BLS	EDS	NDV	MG	MS	CAV	MDV
54	Blood	21	33	Y	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
55	Blood	21	33	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
58	Blood	21	33	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
87	Spleen	21	40	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
89	Litter	21	40	Y	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	+
95	Spleen	21	47	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
96	Spleen	21	47	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
105	Spleen	21	50	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
109	Litter	21	50	Y	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
110	Spleen	21	51	Y	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Percentage of total					40%	0%	0%	0%	0%	10%	0%	10%	0%	0%	0%	0%	0%	0%	30%	100%

(AEV = avian encephalomyelitis virus, AI = avian influenza, AL = avian leukosis, IBD = infectious bursal disease virus, IB = infectious bronchitis, Reo = reovirus, REV = reticuloendotheliosis virus, FAV8 = fowl adenovirus serotype 8, ST = *Salmonella typhimurium*, BLS = big liver and spleen disease, EDS = egg drop syndrome 1976, NDV = Newcastle diseasevirus, MG = *Mycoplasma gallisepticum*, MS = *Mycoplasma synovium*, CAV = chicken anaemia virus, MDV = Marek's disease virus).

Table 6.11 Day 56 serological results for broilers

Treatment	Age at treatment	# of samples (n)	AEV	AI	AL	IBDV	IB	Reo	REV	FAV8	ST	BLS	EDS	NDV	MG	MS	CAV
Buffer	Day old	4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Spleen	Day old	4	25%	0%	0%	0%	0%	50%	0%	0%	0%	0%	0%	0%	0%	0%	25%
Brain	Day old	4	0%	0%	0%	0%	0%	0%	0%	75%	0%	0%	0%	0%	0%	0%	75%
Blood	Day old	4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	50%
Litter	Day old	4	0%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	50%
Buffer	21 days	4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Spleen	21 days	4	25%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%
Brain	21 days	3	33%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	67%
Blood	21 days	4	25%	0%	0%	0%	0%	0%	0%	25%	0%	0%	0%	0%	0%	0%	75%
Litter	21 days	4	50%	0%	0%	0%	0%	75%	0%	100%	0%	0%	0%	0%	0%	0%	50%
Mean titre (±SE) positives			468±19	n/a	n/a	n/a	n/a	2759± 1362	n/a	1.32±0.23*	n/a	n/a	n/a	n/a	n/a	n/a	0.37±0.04*[ǂ]
Mean titre (±SE) negatives			180±18	0.073± 0.001*	72±19	55±9	58±10	97±18	21±5	0.08±0.01*	138±21	52±8	0[£]	2±0.2	98±20	117±19	0.76±0.02*[ǂ]
Effect of challenge (P=)			0.62	1	1	1	1	0.12	1	**<0.001**	1	1	1	1	1	1	**0.04**
Effect of age at challenge (P=)			0.06	1	1	1	1	0.84	1	0.43	1	1	1	1	1	1	0.07

*Results reported as O.D. readings as titre's not available from test, ǂCAV ELISA is a blocking ELISA which is a competitive assay, £Raw data reported from EDS HI test as titre's not available

(AEV = avian encephalomyelitis virus, AI = avian influenza, AL = avian leukosis, IBD = infectious bursal disease virus, IB = infectious bronchitis, Reo = reovirus, REV = reticuloendotheliosis virus, FAV8 = fowl adenovirus serotype 8, ST = *Salmonella typhimurium,* BLS = big liver and spleen disease, EDS = egg drop syndrome 1976, NDV = Newcastle diseasevirus, MG = *Mycoplasma gallisepticum,* MS = *Mycoplasma synovium,* CAV = chicken anaemia virus).

Table 6.12 Day 56 serological results for SPF chickens

Treatment	Age at treatment	# of samples (n)	AEV	AI	AL	IBDV	IB	Reo	REV	FAV8	ST	BLS	EDS	NDV	MG	MS	CAV
Spleen	5 days	3	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	67%
Brain	5 days	3	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	33%
Blood	5 days	3	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	33%
Litter	5 days	3	0%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	33%
Buffer	5 days	3	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Spleen	26 days	3	0%	0%	0%	100%	0%	0%	0%	33%	0%	0%	0%	0%	0%	0%	100%
Brain	26 days	3	0%	0%	0%	33%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	33%
Blood	26 days	3	0%	0%	0%	0%	0%	33%	0%	0%	0%	0%	0%	0%	0%	0%	67%
Litter	26 days	3	33%	0%	0%	100%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	67%
Mean titre (±SE) positives			561	n/a	n/a	5675±1284	n/a	523	n/a	1.65±0.31*	n/a	n/a	n/a	n/a	n/a	n/a	0.42±0.04*‡
Mean titre (±SE) negatives			158±16	0.067±0.001*	24±10	20±9	39±11	43±11	7±3	0.06±0.01*	44±17	39±6	0£	2±0.2	110±40	101±13	0.87±0.04*‡
Effect of challenge (P=)			1	1	1	**<0.001**	1	1	1	**<0.001**	1	1	1	1	1	1	0.24
Effect of age at challenge (P=)			0.53	1	1	**0.005**	1	0.52	1	0.52	1	1	1	1	1	1	0.2

*Results reported as O.D. readings as titre's not available from test, ‡CAV ELISA is a blocking ELISA which is a competitive assay, £Raw data reported from EDS HI test as titre's not available

(AEV = avian encephalomyelitis virus, AI = avian influenza, AL = avian leukosis, IBD = infectious bursal disease virus, IB = infectious bronchitis, Reo = reovirus, REV = reticuloendotheliosis virus, FAV8 = fowl adenovirus serotype 8, ST = *Salmonella typhimurium*, BLS = big liver and spleen disease, EDS = egg drop syndrome 1976, NDV = Newcastle diseasevirus, MG = *Mycoplasma gallisepticum*, MS = *Mycoplasma synovium*, CAV = chicken anaemia virus).

6.3.5.2 Other Diseases

Serological tests were conducted on serum extracted from broiler and SPF chickens on Expt d56 for a range of known poultry diseases (Table 6.3). Results of these tests for broilers are outlined in Table 6.11 and for SPF chickens in Table 6.12. Both tables report the percentage of birds seropositive for each disease by challenge material and age at challenge. Serum samples selected for analysis represent at least 33% of the total broiler population for each challenge and age at challenge combination and at least 60% of the same for SPF chickens. Additionally, mean (±SE) titres are provided for positives (where present) and negatives for each test. For tests from which titres could not be derived, the OD was reported instead, or for the egg drop syndrome (EDS) HI test, raw data were reported. Effects of both challenge and age at challenge are reported.

Sham-challenged chickens were consistently negative for every agent tested in both broiler and SPF populations. A portion of broilers seroconverted to AEV from each challenge material with greater numbers in the later challenged groups. This same result was not reflected in SPF chickens with only one chicken challenged with litter on Expt d21 being seropositive.

There was no serological evidence of infectious bursal disease virus (IBDV) in any of the tested broiler chickens; however, this was not the case for SPF chickens. IBDV was transferred to SPF chickens via each challenge material with significant effects of both challenge ($P < 0.001$) and age at challenge ($P = 0.005$). The later challenged SPF chickens had more seropositive chickens and the spleen inoculum was the most effective means of transmitting the agent.

Seroconversion to reovirus (Reo) (viral arthritis/tenosynovitis) was evident following spleen and litter challenge in broiler chickens and blood challenge in one SPF chicken. Seroconversion to fowl adenovirus serotype-8 (FAV8) resulted from all challenge materials in broiler chickens. All SPF chickens tested that were exposed to litter where seropositive to FAV8 as was one SPF chicken challenged with spleen. There was a significant effect of challenge material ($P < 0.001$) on seroconversion to FAV8 in both broiler and SPF chickens whereby all litter challenged broiler and SPF chickens were seropositive to FAV8, varying levels of brain, blood and spleen challenged chickens were FAV8 seropositive and all sham treated control broiler chickens were seronegative to FAV8.

Seroconversion to CAV occurred in chickens in all challenge and age at challenge combinations in both broiler and SPF chickens with a significant effect of challenge ($P = 0.04$) in broilers. Of the samples tested there was no evidence of seroconversion to the agents implicated in AI, avian leukosis, reticuloendotheliosis, *Salmonella typhimurium*, big liver and spleen disease, EDS, ND, *Mycoplasma gallisepticum* and *Mycoplasma synoviaue.* Table 6.10 displays serological results as

positive (+) or negative (-) for the same agents from all broilers that developed the APS from which serum samples could be obtained. Serconversion to AEV was observed in 40% of cases and to CAV in 30% of cases. Evidence of seroconversion to FAV8 and Reo was evident in one bird.

6.3.6 Molecular Diagnostics

6.3.6.1 PCR for the detection of MDV

Standard PCR's for MDV1 using the two primer sets outlined in Table 6.4 were performed on DNA extractions from a selection of tissues as outlined in Table 6.13. This test, unlike the MDV ELISA, is serotype-specific and excludes the vaccine virus HVT (MDV serotype 3). All experimental samples tested (Table 6.13), which included an equal number of samples from HVT vaccinated and unvaccinated broilers, were negative for MDV1 as determined by gel electrophoresis. Figures 6.9 and 6.10 depict the typical findings using agarose gel electrophoresis to analyse amplified DNA products.

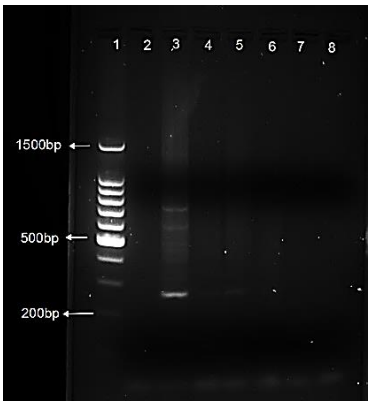

Figure 6.9 Agarose gel analysis of amplified DNA using pp38 primer set. 1: 100 bp DNA ladder; 2: No template control. 3; DNA extracted from MDV1 (Rispens) vaccinated broiler positive control, showing most intense band of 264 bp; 4-6: DNA extracted from broilers with the APS (spleens); 7: DNA extracted from spleen challenge material; 8: DNA extracted from blood challenge material.

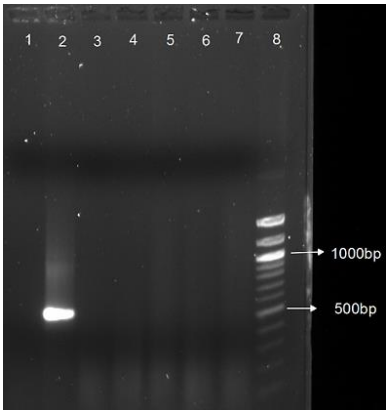

Figure 6.10 Agorose gel analysis of meq amplified DNA using BCH342/SJW1 primer set. 1: No template control; 2: DNA extracted from MDV1 (Rispens) vaccinated broiler positive control showing 577 bp band; 3: DNA extracted from spleen challenge material; 4: DNA extracted from blood challenge material; 5-7: DNA extracted from broilers with the APS (spleens); 8: 100 bp DNA ladder.

Table 6.13 Summary of DNA samples used for MDV PCR and qPCR

Breed/ID	Challenge material	Vacc. status (Y/N)	Age at challenge	Extraction tissue	Total samples
Day 56 Survivors					
Broiler	Buffer	Yes	21	Spleen	2
Broiler	Buffer	Yes	21	Spleen	2
Broiler	Spleen	Yes	21	Spleen	2
Broiler	Spleen	Yes	21	Spleen	2
Broiler	Blood	Yes	21	Spleen	2
Broiler	Blood	Yes	21	Spleen	2
Broiler	Litter	Yes	21	Spleen	2
Broiler	Litter	Yes	21	Spleen	2
SPF	Spleen	No	21	Spleen	3
SPF	Blood	No	21	Spleen	3
SPF	Litter	No	21	Spleen	3
SPF	Buffer	No	1	Spleen	3
Broilers with the APS					
55	Blood	No	21	Spleen	1
55	Blood	No	21	Brain	1
57	Blood	Yes	21	Spleen	1
57	Blood	Yes	21	Brain	1
58	Blood	No	21	Spleen	1
58	Blood	No	21	Brain	1
87	Spleen	Yes	21	Spleen	1
87	Spleen	Yes	21	Brain	1
89	Litter	No	21	Spleen	1
89	Litter	No	21	Brain	1
95	Spleen	Yes	21	Spleen	1
95	Spleen	Yes	21	Brain	1
Challenge Material					
Blood					1
Spleen					1
Brain					1
Total DNA extractions					**43**

6.3.6.2 Real-time PCR for the detection of MDV

The same set of samples (Table 6.13) were assayed by qPCR for MDV1 as described in 6.2. All samples selected tested negative for the presence of MDV1 DNA.

A selection of DNA extracted from the spleens of broiler and SPF chickens and the initial spleen and blood challenge material (Table 6.14) were amplified via the HVT qPCR assay as described in 6.2. The reason for conducting this assay was to determine whether HVT infection was causing birds to be MDV seropositive. Results from this assay further confirm the effectiveness of HVT vaccination of broilers given 100% of positive samples for buffer inoculated HVT vaccinated broilers and 100% of negative samples for buffer-inoculated unvaccinated broilers (Table 6.14). Both of the spleen and blood challenge materials were positive for HVT. All of the spleen and blood inoculated broilers were HVT positive despite vaccination status. A portion of spleen inoculated and all blood inoculated SPF chickens were HVT positive. All litter-challenged and HVT-vaccinated broilers were HVT positive; however, litter-challenged and unvaccinated chickens (broilers and SPF) were HVT negative. These results confirm that seropositivity to MDV

was from HVT infection, either from vaccination or transferred via the spleen or blood challenge materials.

Table 6.14 Summary of HVT qPCR results by treatment

Breed	Challenge	Age at challenge	HVT Vaccination Status	Number of Samples	Samples Positive (%)
Broiler	Buffer	21	Vaccinated	2	100%
Broiler	Buffer	21	Unvaccinated	2	0%
Broiler	Spleen	21	Vaccinated	2	100%
Broiler	Spleen	21	Unvaccinated	2	100%
Broiler	Blood	21	Vaccinated	2	100%
Broiler	Blood	21	Unvaccinated	2	100%
Broiler	Litter	21	Vaccinated	3	100%
Broiler	Litter	21	Unvaccinated	1	0%
SPF	Spleen	21	Unvaccinated	3	33%
SPF	Blood	21	Unvaccinated	3	100%
SPF	Litter	21	Unvaccinated	3	0%
SPF	Buffer	1	Unvaccinated	3	0%
Spleen challenge material (Table 6.2)				1	100%
Blood challenge material (Table 6.2)				1	100%

6.3.6.3 RT-PCR for the detection of AEV

Efforts to detect AEV in tissue samples from affected chickens were made as such was included in the differential diagnosis list for the APS (Chapter 4). An RT-PCR was run in order to detect AEV RNA in the tissues of affected and unaffected chickens. RNA was extracted from a selection of spleens and brains from broiler chickens demonstrating the APS and AEV seropositivity, spleens from inoculated broiler chickens that were clinically normal and AEV seropositive, spleens from inoculated broilers that were clinically normal and AEV seronegative and spleens from buffer-inoculated and AEV seronegative broiler and SPF chickens for the RT-PCR as described in 6.2. All reactions were analysed via agarose gel electrophoresis and were found to be negative for AEV. Figure 6.11 depicts the typical findings from these analyses. RNA extracted from AEV vaccine was put through the same RT-PCR system and used as a positive control sample.

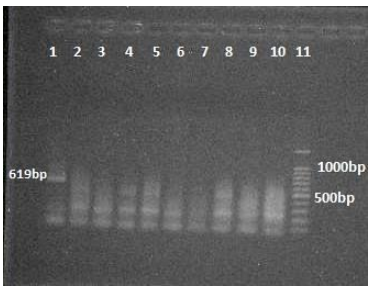

Figure 6.11 Agarose gel analysis for RT-PCR products using the AEV MKAE1FWD/MKAE2REV primer set. 1: RNA extracted from AE vaccine, showing a 619 bp DNA fragment. 2: RNA extracted from brain of a broiler with the APS and AE seropositive. 3: RNA extracted from spleen of a broiler with the APS and AE seropositive. 4: RNA extracted from brain of a broiler with the APS and AE seropositive. 5: RNA extracted from spleen of a broiler with the APS and AE seropositive. 6: RNA extracted from spleen of buffer-challenged and AE seronegative broiler. 7: RNA extracted from spleen of buffer-challenged and AE seronegative broiler. 8: RNA extracted from brain of a broiler with the APS and AE seronegative. 9: RNA extracted from spleen of a broiler with the APS and AE seronegative. 10: RNA extracted from spleen of a buffer challenged and AE seronegative broiler. 11: 100 bp DNA ladder.

6.3.6.3 RT-PCR for the detection of flaviviruses

Efforts to detect flaviviruses in tissue samples from affected chickens were made as they were included in the differential diagnosis list for the APS (Chapter 4). As was discussed in Chapter 4, of the 12 arboviral families, only viruses in the *Togaviridae* and *Flaviviridae* families are known to affect domestic poultry. Diagnostics herein focussed on the *Flaviviridae* family given the recent outbreak of neurological disease in equine populations in eastern Australia from Kunjin virus (Frost *et al.*, 2012) which is a member of the *Flaviviridae* family.

All reactions were analysed via agarose gel electrophoresis and were found to be negative. Figure 6.12 depicts the typical findings from these analyses. RNA extracted from Palm Creek virus was put through the same RT-PCR system and used as a positive control sample.

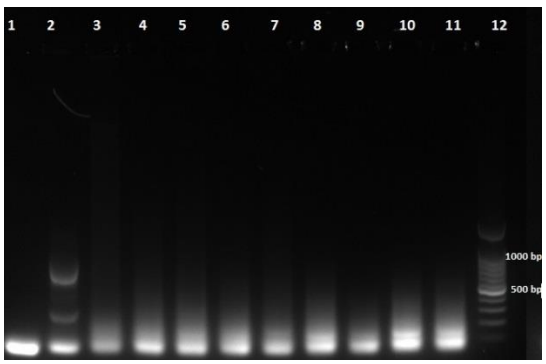

Figure 6.12 Agarose gel analysis of RT-PCR products using the pan-flavivirus FUC/cFD3 primer set. 1: NTC. 2: RNA extracted from Palm Creek virus showing an 845 bp DNA fragment. 3-7: RNA extracted from spleens from broilers with the APS. 8-11: RNA extracted from brains from broilers with the APS. 12: 100 bp DNA ladder.

6.4 Discussion

From this experiment it is evident that the APS as observed in broiler chickens in the field is reproducible in a controlled experimental setting and the clinical signs observed in affected experimental chickens closely resemble that of affected field chickens. The conditions under

which the APS was reproduced were quite specific, requiring inoculation of 21-day-old broilers with either splenocytes or blood via intra-abdominal injection, or by exposing them to litter harvested from field sheds experiencing the disease. The APS was not reproduced in chickens challenged at hatch or in SPF chickens challenged at either hatch or 21 days. With these findings the first hypothesis of this experiment is accepted in part, acknowledging the APS was not reproduced from brain inocula, broilers challenged at day old or in SPF chickens.

The experiment showed that HVT vaccination did not provide protection against the APS and additionally MDV1 could not be isolated from chickens demonstrating the APS. Thus MDV is not the causative agent of the APS so the second hypothesis is accepted.

The third hypothesis of the experiment is accepted in part, as the age of exposure correlated with the development of the APS in broilers but not in SPF chickens, in which the syndrome could not be produced.

The results of this experiment suggest that the APS is caused completely or in part by an infectious agent/s. The agent/s is likely associated with lymphoid cells because disease was transmitted by inoculation with splenocytes. The agent/s is also present in blood, in a cell-associated or free state. The ability to transmit the disease by the exposure of chickens to litter suggests that the agent/s is shed by infected birds and persists in litter material.

The development of disease in broilers challenged at 21 days of age and not in those challenged at day old could be a result of MAb to the infectious agent/s providing protection at this earlier age. As the group which were exposed to contaminated litter at hatch were potentially continuously exposed to the putative agent throughout their life via the litter and did not develop the APS, adaptive immunity likely developed from the early challenge while protected. However in relation to MAb, the syndrome was not induced in SPF chickens, presumably free of such MAb. Thus SPF chickens were not susceptible to the syndrome, which suggests that breed or genotype may play a role in susceptibility to the disease. The smaller number of SPF chickens in each treatment group (5) may affect this interpretation given that clinical disease was only manifested in 35% of chickens in each of the affected blood and spleen challenge groups and 10% of the affected litter challenged group. Nevertheless we would expect to have seen cases in relevant SPF groups given these ratios. Another possible explanation for absence of disease in the SPF chickens is the phenomenon of antibody-dependant enhancement (ADE) of infectivity, whereby the presence of antibody-coated viruses (immune-complexes) results in enhanced infection of monocytes with Fcγ receptors. ADE is an important aspect of some viral infections such as dengue fever in humans (Kliks *et al.*, 1989) and porcine reproductive and respiratory syndrome virus in pigs (Mateu &

Diaz, 2008). However, the apparent antibody protective effect noted in day old challenged broilers in this experiment is not consistent with ADE.

Findings from this experiment highlight that male broilers are more susceptible to the APS than are females; however, females can certainly succumb to it. This finding is consistent with field observations of greater mortalities in male portions of flocks with the APS (Chapter 5).

The early stages of the paralysis syndrome as described in 6.3 can be easily confused with lameness and thus diagnosis based on clinical signs relies on the progression to later stages of paralysis, when the bird exhibits flaccid paralysis of the neck, in addition to other signs as described in Table 6.6.

The principal histopathological lesions observed in the brains of chickens euthanased with APS were those of vasculocentric encephalitis evidenced by perivascular cuffing of mononuclear cells and often the progression to vasculitis with endothelial hypertrophy and perivascular oedema. Similar changes were noted in spinal cords in addition to Wallerian degeneration. The Wallerian degeneration could be attributed to vasogenic oedema association with vasculitis causing trauma to neurones. The inflammatory changes observed in the brain of affected chickens were consistent with field observations (Chapter 4) and cannot be considered pathognomonic of any known disease of broiler chickens. However, as discussed in Chapter 4, similar pathology is observed in MD, AE and flaviviral infections of poultry which were considered as differential diagnoses prior to conducting the experiment set out in this chapter. The histopathological lesions observed in some of the affected birds from this experiment were subtle and thus could be easily overlooked by the untrained eye. In this case the pathologist was blinded in analysing brain sections from affected and unaffected birds. Nevertheless, there was complete correlation between birds demonstrating clinical signs of the APS and the presence of inflammatory brain pathology. The subtleness characterising some of the lesions in cases from this experiment is not too different to the other viral diseases inducing inflammatory brain pathology as previously mentioned.

The neurological characteristics of chickens affected with the APS were consistent with those observed associated with affected field chickens as set out in Chapter 4. Chickens affected with the APS in this experiment demonstrated normal functioning cranial nerves, normal spinal reflexes (vent-sphincter, pedal-flexor and wing-withdrawal), normal postural reactions and functioning nociception which isolates the location of lesions in these chickens to within the forebrain (cerebrum and diencephalon). This is consistent with histopathology being detected in the cerebrum of affected chickens. The presence of normal postural reactions is not consistent with the pathology observed in spinal cords given that ascending sensory proprioceptive pathways

in the spinal cord are located in the outermost regions thus should have reduced functionality in the event of pressure associated trauma which could be expected from the observed inflammatory pathology. This suggests that the observed spinal cord pathology is not contributing to the clinical signs observed.

In further reproduction experiments relating to the APS in broiler chickens CNS histology should be studied in inoculated but clinically normal birds to assess whether similar histopathology is present. The ability to correlate severity of histopathological lesions with stages of the APS as described in 5.3 would be an interesting addition to these findings but would require improved ability to differentiate the early signs from lameness. Furthermore, additional spinal cord histology from paralysed and inoculated but clinically normal birds needs to be assessed to better define the spinal cord lesions associated with the disease. Unfortunately CNS histology was not able to be examined from inoculated SPF chickens from this experiment due to lack of sampling. Such studies could be of value if considering potential variations in genetic susceptibility to this syndrome.

A significant difference in bodyweights was noted in litter challenged chickens compared to sham-challenged chickens with challenge occurring at day old, and weights recorded 14 dpc. The reduced early growth rates observed is likely associated with early challenge to a wide array of infectious agents present in litter (compared to blood, spleen and brain). This difference was non-significant at 56 days of age, at which point the effect of challenge material was insignificant. This finding is consistent to what has been observed in the field (Chapter 5) as the presence of the APS has not been associated with poor performance (measured by bodyweight) of the flock, but rather has been associated with higher early bodyweights.

The attention paid to relative immune organ weights in this experiment was in order to assess any likely immunological response associated with the APS as measured by increases or decreases in bursa of Fabricius and spleen size. In the Expt d56 broiler population there was not a clear trend in relation to relative bursal weight and challenge material and age at challenge. The mean relative bursal weights of the 21 day-old litter challenged broiler group was greatest, which would be consistent with the diverse range of agents to which these chickens would be immunologically challenged with via the litter material used. In the Expt d56 SPF chicken population, those chickens challenged with litter at 21 days of age had significantly lower mean bursal weights relative to all other groups except spleen challenged groups. These findings are consistent with the evidence of seroconversion to infectious bursal disease virus in all tested chickens in these groups (litter challenged at 21 days; spleen challenged at both day old and 21 days).

In the Expt d56 broiler population, the sham-challenged broilers were at the lower end when considering mean relative spleen weights, which would be consistent with the challenged groups responding immunologically to challenge with greater spleen size. There was not a clear trend in relative spleen weights between the challenged groups and ages at challenge. In the Expt d56 SPF chicken population, the litter challenge groups had significantly lower mean relative spleen weights compared to the other challenged groups, of which all tested chickens were seropositive for FAV and all of the 21 day of age challenge group were positive for IBDV.

The interpretation of MD serology was complicated by half of the broilers in the experiment being HVT vaccinated, the blood and spleen inocula being from broilers that were HVT vaccinated and the ELISA procedure being incapable of distinguishing between the different MDV serotypes. Nevertheless it established that only birds receiving HVT directly via vaccination or potentially indirectly by inoculation of tissues from HVT vaccinated birds seroconverted. HVT-specific real-time qPCR revealed that the spleen and blood inocula were HVT positive and the virus was transmitted to most chickens into which either was inoculated, as outlined in Table 6.14. Challenge with litter was not effective in transmitting HVT to either broilers or SPF chickens. The failure to detect MDV1 in any of the standard or qPCR procedures employed which are known to be sensitive detectors of MDV presence, coupled with the lack of an effect of HVT vaccination on incidence of the APS largely rules out MDV as a causative agent in the APS.

From serological results, there is no clear link between chickens with the APS and a particular known disease. It is evident that CAV was present in each challenge material used and perhaps most interesting that CAV was transmitted to experimental chickens via the brain inoculum. The most likely explanation is that this virus was present within the blood and/or monocytes within the brain tissue that was used, which is consistent with the known resilience of CAV.

Evidence of AEV was not detected in a selection of tissues via RT-PCR. This suggests that the involvement of AEV in the APS is unlikely. This is consistent with not all paralysed chickens being seropositive to the virus, and some non-paralysed chickens being positive. Similarly, evidence of flaviviruses was not detected in a range of tissue samples which largely rules out flaviviral disease as a differential diagnosis for the APS.

As a result of the findings of this experiment, further experimental investigations into the APS should utilise the experimental model derived from this experiment (challenge of 21 day old broiler chickens with spleen, blood and/or litter). Future investigations to identify the likely infectious aetiological agent/s associated with the APS could use the filtration of challenge material in order to identify the likely size range of the infectious agent, or alternatively the more

recent technology of whole genome next-generation sequencing (Chapter 9) to detect viruses and bacteria in biological samples. Additional aspects, such as testing challenge material in different broiler chicken genotypes (e.g. Cobb 500) and attempting to better describe the clinical syndrome and histopathological lesions would be useful. Further passage of blood, spleen and litter collected from experimentally infected chickens and being able to induce the APS with such would be useful in confirming the infectious nature of this disease.

The high level of hygiene and sanitation standards used in the region affected with the APS (Chapter 4) would suggest that any challenge from any infectious agent/s associated with the APS would occur later in life. Given the likely protective effects against the APS from MAb reported in this Chapter, future investigations could be directed at exploring the relationship between earlier challenge and MAb protection because it is possible that challenge at an age at which there is still some maternal antibody protection may actually be protective against the APS if these chickens are able to develop protective adaptive immunity. If this was found to be the case, strategies such as litter re-use could be quickly implemented on farm to reduce incidences of the APS.

6.5 Conclusion

The APS under investigation could be reproduced in broiler chickens challenged at 21 days of age with spleen or blood harvested from affected chickens and injected intra-abdominally, or exposed to litter collected from affected sheds. Male broilers were more susceptible to the disease and HVT vaccination did not provide protection. The experimental results do not shed light on what the likely causative agent(s) may be, but are strongly suggestive of the non-involvement of some known viral causes of neurological disease in chickens including MDV, AEV, and flaviviruses.

Chapter 7: Further experimental investigations into an acute paralysis syndrome of broiler chickens: the protective effects of exposure to contaminated litter at placement and infectivity of first chicken passage and field harvested material

7.1 Introduction

The three experiments described in this chapter were designed to follow up on findings from the experiment reported in Chapter 6. The experimental reproduction model found to be effective in Chapter 6 requiring the challenge of broiler chickens at 21 days of age with potentially infective material was used. Male chickens were found to be more susceptible to the acute paralysis syndrome (APS) (Chapter 6) and thus were solely used in the experiments reported in this chapter in order to maximise the chances of reproducing the APS.

The inability to reproduce the APS in chickens challenged at day-old in the experiment reported in Chapter 6 (13-C-REP1) could be a result of protective maternal antibody (MAb). Chickens are protected by MAb against disease from a range of infectious agents, such as avian encephalomyelitis virus (AEV) (Westbury & Sinkovic, 1978a) and CAV (Otaki *et al.,* 1992). Typically MAb levels cease being protective from 10-14 days (Patterson *et al.,* 1962) and it is commonly believed that protection by MAb to an infectious agent cannot be guaranteed after two half-lives of that antibody. Typical half-lives off common disease causing agents of poultry range from 3.8 ± 0.5 days in the case of infectious laryngotracheitis virus to 7.0 ±0.4 days of CAV (Gharaibeh & Mahmoud, 2013). The first experiment described in this chapter (experiment 1) was designed to test the hypothesis that chickens exposed to contaminated shed litter at placement (day of hatch) and challenged at 21 days of age would show a significantly reduced susceptibility to the APS compared to chickens which did receive the early exposure prior to challenge. This hypothesis is consistent with observations by Chu & Rizk (1975) associated with live Newcastle disease (ND) virus vaccine administration early in life. They found that MAb acted to decrease the severity of the vaccine reaction but did not completely prevent immunity from developing to the virus. In the design of experiment 1 herein, by placing chickens on contaminated litter it was predicted that they would be protected against the APS. This protection may be provided from protective MAb and the subsequent development of adaptive immunity.

Whole blood and litter were selected as challenge materials for experiment 1 as they were effective in inducing the APS in the experiment reported in Chapter 6 and were relatively easy to harvest and store. Given the APS could be induced after inoculation with spleen cells or blood and if the APS is caused by infectious agent/s, all other host, pathogen and environmental considerations being held constant it could be reasonably expected that the APS could be induced with first chicken passage spleen and blood challenge material. The second experiment reported in this chapter (experiment 2) was designed to test the hypothesis that whole blood and spleen cells harvested from chickens affected with the APS from the experiment reported in Chapter 6 would induce the APS in broilers on subsequent chicken passage. The purpose in testing this prediction was to establish whether the putative agent/s maintain pathogenicity through serial biological passage which is an important consideration in experimental reproduction models (Evans, 1976).

In order to maintain the ability for experimental reproduction of the APS a sufficient stock of challenge material is required. Unfortunately there was not any residual challenge material from the experiment report in Chapter 6. The third experiment reported in this chapter (experiment 3) was designed firstly to test the infectivity of three sets of separately harvested challenge material from the field. If the APS is caused by infectious agent/s, it could be reasonably expected that a greater incidence of the APS would be observed with increasing doses of the challenge material. Experiment 3 was also designed to test the prediction that if infectivity was confirmed for a specific batch of challenge material, that the prevalence of APS would be greater in chickens challenged with a higher dose of the challenge material compared to the standard 200 µL intra-abdominal (IA) injection as used for the experiment reported in Chapter 6.

7.2 Materials and Methods

7.2.1 Experimental design

Experiment 1 (13-C-REP2) was approved by the University of New England (UNE) Animal Ethics Committee (AEC13-101), commenced 27 August 2013 and was terminated in 8th October 2013. In order to test the protective effects of early exposure to contaminated litter a 2 x 2 factorial design with two replicates was used (Table 7.1) utilising 160 unvaccinated male Ross broiler chickens with 20 chickens being placed in each of eight isolators. The two experimental factors were:

- Litter exposure at placement: two treatments were used, exposure to contaminated shed litter in the isolator at placement (mixed with shavings at rate of 1 kg/isolator) and no exposure.

- Challenge material: two treatments were applied at 21 days of age (21d). One treatment consisted of intra-abdominal (IA) inoculation with 200 µL of whole blood harvested from affected chickens from a field case (field blood A) and exposure to shed litter harvested from the same case (mixed with existing litter at rate of 1 kg/isolator). IA inoculation with 200 µL of buffer (sterile vaccine diluent) was used as a sham treatment.

Table 7.1 Experiment 1 design

Isolator ID	Litter exposure at placement (Y/N)	Challenge material (at 21 days of age)	Number of chickens placed (n)
1	N	Buffer	20
2	N	Blood + contaminated litter	20
3	Y	Buffer	20
4	Y	Blood + contaminated litter	20
5	N	Buffer	20
6	N	Blood + contaminated litter	20
7	Y	Buffer	20
8	Y	Blood + contaminated litter	20

Experiment 2 (13-C-REP3) was approved by the UNE Animal Ethics Committee (AEC13-101) and run concurrent with experiment 1. In order to test the infectivity of first chicken passage spleen and blood material three challenge treatments were used (Table 7.2) utilising 80 unvaccinated male Ross broiler chickens with 20 chickens being placed in each into each of four isolators. The three challenge treatments were:

- Buffer (sterile vaccine diluent) as sham treatment – 200 µL IA injection.
- Blood (200 µL IA injection) harvested from chickens affected with the APS reported in Chapter 6.
- Spleen cells (200 µL IA injection) harvested from chickens affected with the APS reported in Chapter 6.

Table 7.2 Experiment 2 design

Isolator ID	Challenge material (21 days of age)	Number of chickens placed (n)
1	Buffer	20
2	Buffer	20
3	Blood (1st chicken passage)	20
4	Spleen (1st chicken passage)	20

Experiment 3 (13-C-REP4) was approved by the UNE Animal Ethics Committee (AEC 13-148), commenced 7 November 2013 and was terminated on 24 December 2013. In order to test the infectivity of various challenge materials and any dose effect of challenge, a 4 x 2 factorial design was used (Table 7.3) utilising 40 unvaccinated male Ross broiler chickens housed in two isolators. The two experimental factors were:

- Challenge material: four challenge treatments were used including sterile phosphate buffered saline (PBS) as a sham treatment, whole blood sourced from affected chickens from a field case (field blood A), whole blood sourced from affected chickens from a separate field case (field blood B) and first chicken passage whole blood harvested from chickens affected with the APS in the experiment reported in Chapter 6, all administered by IA injection.
- Challenge dose: two levels were used, 200 µL and 1,000 µL.

Table 7.3 Experiment 3 design

Isolator ID	Challenge material	Dose	Number of chickens (n)
1	PBS	200 µL	5
1	Blood (field A)	200 µL	5
1	Blood (field B)	200 µL	5
1	Blood (1st passage)	200 µL	5
2	PBS	1,000 µL	5
2	Blood (field A)	1,000 µL	5
2	Blood (field B)	1,000 µL	5
2	Blood (1st passage)	1,000 µL	5

7.2.2 Animals & Management

Ross broiler chickens (males) were hatched by the hatchery supplying most of the broiler farms in the affected region (Chapter 5) and were transported by air-freight to UNE and placed into isolators on the day of hatch (20 chickens per isolator) which was the day of commencement (Expt d0) of each experiment. For experiments 1 and 2 litter flooring trays were installed into isolators (Chapter 3) with white wood shavings placed during isolator construction. Litter trays were not installed into isolators for experiment 3 instead the flooring was perforated stainless steel (Chapter 3). The feed loaded into each isolator for experiment 3 was sourced from the same feed mill that supplies all broiler growing farms in the region experiencing the APS (Chapter 5). This feed consisted of broiler starter and grower rations. Commercially available rations sourced locally to UNE were utilised for experiments 1 and 2. In each experiment, chickens were visually inspected twice daily.

7.2.3 Treatments

The description of each of the challenge materials and their doses used for each experiment are provided in Table 7.4. The detail relevant to the processing and storage of blood and spleen material prior to challenge is provided in Chapter 3. Challenge with blood or spleen materials occurred via IA injection at 21d. Contaminated litter was spread through allocated isolators and mixed with the existing litter at 21d for experiment 1.

Table 7.4 Challenge materials and doses used for each experiment

Expt	Material	Dose	Source
1	Buffer	200 µL	Sterile vaccine diluent, Merial Select Inc., Gainesville, USA, Lot: DP115.
	Whole blood (field blood A)	200 µL	Harvested from several chickens with APS from one field case (July 2013) and different to that from which material was harvested for the experiment reported in Chapter 6. Cryopreserved at UNE as per Chapter 3.
	Shed litter	1 kg/isol.	Sourced from same field case (July 2013). Stored at UNE at -20°C.
2	Buffer		Sterile vaccine diluent, Merial Select Inc., Gainesville, USA, Lot: DP115.
	Whole blood (1^{st} passage)	200 µL	Harvested from several chickens with APS from the experiment reported in Chapter 6. Cryopreserved at UNE as per Chapter 3.
	Spleen cells (1^{st} passage)	200 µL ($4.3x10^6$ cells/mL)	Harvested from several chickens with APS from the experiment reported in Chapter 6. Cryopreserved at UNE as per Chapter 3.
3	Buffer	200 µL or 1,000 µL	Sterile PBS: 1-To 800 mL of distilled water added 8 g of NaCl + 0.2 g KCl + 1.44 g Na_2HPO_4 + 0.24 g KH_2PO_4. 2-Adjusted pH to 7.4 with HCl. 3-Adjusted volume to 1 L with distilled water. 4-Sterilised by autoclaving.
	Whole blood (field blood A)	200 µL or 1,000 µL	Field blood A: harvested from several chickens with APS from one field case (July 2013) and different to that from which material was harvested for the experiment reported in Chapter 6. Cryopreserved at UNE as per Chapter 3.
	Whole blood (field blood B)	200 µL or 1,000 µL	Field blood B: harvested from several chickens with APS from one field case (October 2013) and different to that from which material was harvested for the experiment reported in Chapter 6. Cryopreserved at UNE as per Chapter 3.
	Whole blood (1^{st} passage)	200 µL or 1,000 µL	1^{st} chicken passage blood: harvested from several chickens with APS from the experiment reported in Chapter 6. Cryopreserved at UNE as per Chapter 3.

7.2.4 Sample collection

7.2.4.1 Experiment 1

Numbers in each isolator were reduced to 12 chickens at 14 days post-challenge (dpc). Chickens removed at 14 dpc were humanely euthanased via cervical dislocation and body weights were recorded. A post-mortem (PM) examination was then performed on each chicken. Spleen and bursal weights were recorded. The experiment was continued until chickens were 42 days of age (Expt d42). At the conclusion of the experiment, chickens were euthanased for subsequent body and immune organ weight measurements and PM examination. Blood was collected from a

cutaneous ulnar vein from five chickens per isolator on Expt d42 into plain serum tubes (BD Vacutainer® ref: 366668) for serum extraction and storage (-20°C).

7.2.4.2 Experiment 2

As per experiment 1.

7.2.4.3 Experiment 3

Blood was collected from 10 chickens at on Expt d0 (terminal) and 14 days of age into plain serum tubes for subsequent serum extraction and storage (-20°C) in order to be able to determine a maternal antibody profile for a selection of agents. Numbers in each isolator were reduced to 12 chickens at 14 dpc, retaining three chickens per challenge/dose combination where numbers remaining permitted. The experiment was continued until chickens were 47 days of age (Expt d47). At the conclusion of the experiment, chickens were euthanased for subsequent body and immune organ weight measurements and PM examination. Blood was collected from a cutaneous ulnar vein into plain serum tubes from all chickens remaining on Expt d47 for subsequent serum extraction and storage. Fixed sections of brain and spinal cord (from each segment) were stored fixed in 10% neutral buffered formalin (100 mL 37% formaldehyde, 900 mL distilled water, 4 g/L NaH_2PO_4, 6.5 g/L Na_2HPO_4, pH 7) from any chicken demonstrating the APS and from a selection of sham-treated controls from the same experiment.

7.2.5 Laboratory techniques

7.2.5.1 Histopathology

Fixed brain and spinal cord sections were sent to the State Veterinary Diagnostic Laboratory (SVDL) for histological evaluation by avian pathologist Dr. Rod Reece. This service was provided by the SVDL at commercial rates.

7.2.5.2 Serology

Serological testing on serum extracted from a selection of chickens in each experiment was performed by BAL. Testing was conducted using commercially available enzyme linked immunosorbent assay (ELISA) kits (Table 7.5) for AEV, infectious bursal disease virus (IBDV), fowl adenovirus serotype 8 (FAV8), reovirus (Reo) and CAV. These tests were selected due to the variable levels of seropositivity to each of these agents in the experiment reported in Chapter 6.

Table 7.5 ELISA kit details for serological tests performed at Birling Avian Laboratories

Agent	Test, Source
Avian encephalomyelitis virus	ELISA, IDEXX Laboratories, Batch: 99-09259 CJ231, Exp: 8/7/2014
Infectious bursal disease virus	ELISA, IDEXX Laboratories, Batch: 99-09260 FJ663, Exp: 11/9/2014
Fowl adenovirus serotype 8	ELISA, Trop Bio Pty Ltd., Batch: 02-019-01, Exp: Oct 2013
Reovirus	ELISA, IDEXX Laboratories, Batch: 99-08702 BJ058, Exp: 7/4/2014
Chicken anaemia virus	ELISA, IDEXX Laboratories, Batch: 99-09264 KH495, Exp: 18/3/2014

7.2.6 Statistical Analysis

Statistical analysis of experimental data was conducted using the software package JMP 10 (SAS Institute Inc., 2012). A significance level of $P < 0.05$ was accepted for all statistical tests and in all cases the individual chicken was the experimental unit. Time to event analyses, quantifying the time taken for birds to die or require euthanasia (due to the APS) following enrolment into the study (in days), were described using Kaplan-Meier survival curves. All chickens that were euthanased at 14 dpc for scheduled measurements and sampling were excluded from such analyses. Relative immune organ weights (bursa of Fabricius and spleen) were expressed as a percentage of bodyweight (relative organ weight = [(organ weight/bodyweight)x100]).

For continuous variables including bodyweight and relative immune organ weights, distributions were assessed for normality and distribution of residuals to assess the need for transformation, from which none was required. Analyses were conducted using a general linear model, fitting the effects of early litter placement, challenge material, challenge dose, early litter placement and challenge material nested within allocated isolator and PM operator, each where applicable. From such analyses least-squares means (LSM) and standard errors are presented and Tukey's HSD test was used to detect differences between more than two means.

Analysis of discrete serological data (positive or negative) was performed using a general linear model with a binomial link function (logistic) fitted for the effects of early litter exposure, challenge material, challenge dose, early litter exposure and challenge material nested within isolator, each where applicable.

7.3 Results

7.3.1 Experiment 1

7.3.1.1 Reproduction of the APS

The APS was not reproduced in any chicken throughout the course of this experiment.

7.3.1.2 Survival

The effects on survival of early exposure to litter (P = 0.20) and challenge material (P = 0.55) were both non-significant (Figure 7.1).

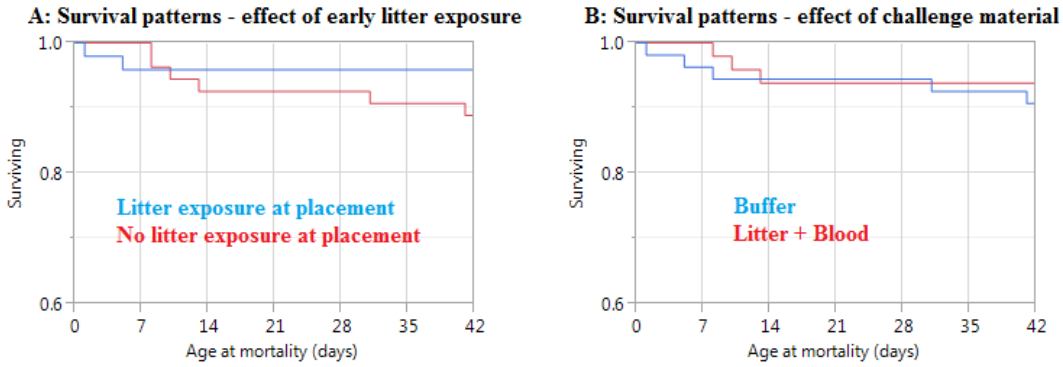

Figure 7.1 Survival patterns of chickens in experiment 1. A: The effect of litter exposure at placement was non-significant (P = 0.20). B: The effect of challenge material was non-significant (P = 0.55).

7.3.1.3 Bodyweights

Mean bodyweight at 35 days of age was 1335±35 g and at 42 days of age was 1832±27 g. Early exposure to litter had a significant effect on bodyweight at 35 days of age (P = 0.03) with the mean bodyweight of those chickens exposed being 10.8 % less than those unexposed (Table 7.6). However, at the conclusion of the experiment the difference in bodyweight was not significant (Table 7.6). These results likely reflect diminished earlier performance from early pathogen challenge, which subsequently was recovered. The effect of challenge material on 35 and 42 day-old bodyweights were non-significant (Table 7.6).

The interaction between the effects of early litter exposure and challenge material on bodyweight was non-significant at both 35 and 42 days of age (Table 7.6, Figure 7.2). Similarly the effect of early litter exposure and challenge material nested within allocated isolator was non-significant at 35 and 42 days of age (Table 7.6).

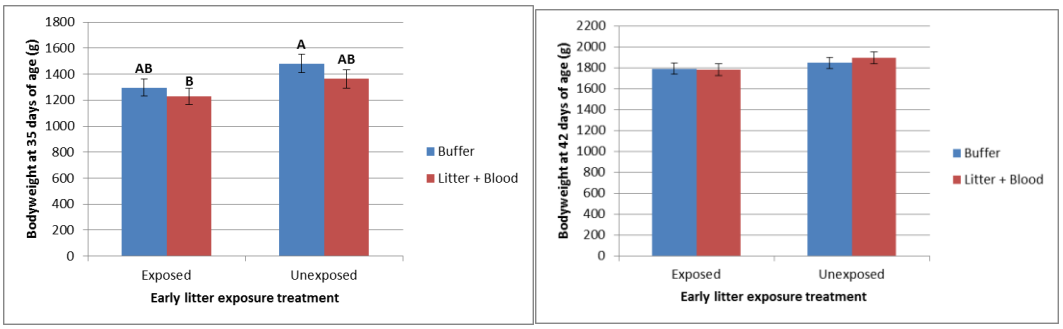

Figure 7.2 Experiment 1. Least-squares means (±SE) for bodyweights by early exposure to litter treatment and challenge material at 35 (left panel) and 42 (right panel) days of age. Where indicated, columns not sharing a common letter differ significantly.

7.3.1.4 Bursa of Fabricius

Early exposure to litter had no significant effect on relative bursal weight at 35 days of age (Table 7.6). However, at 42 days of age early exposure to litter had a significant effect on relative bursal weight (P = 0.01), with relative bursal weights of exposed chickens being 24 % greater than those unexposed (Table 7.6). Challenge material had no significant effect on relative bursal weight at either 35 or 42 days of age (Table 7.6).

The interaction between the effects of early litter exposure and challenge material was non-significant at 35 and 42 days of age (Table 7.6, Figure 7.3). Similarly the interaction between the effects of early litter exposure and challenge material nested within allocated isolator was non-significant at both 35 and 42 days of age (Table 7.6).

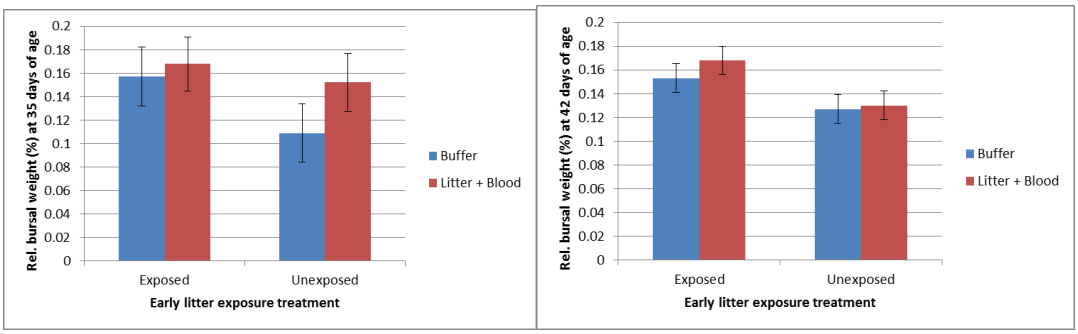

Figure 7.3 Experiment 1. Least-squares means (±SE) for relative bursal weights (% body weight) by early exposure to litter treatment and challenge material at 35 (left panel) and 42 (right panel) days of age. Where indicated, columns not sharing a common letter differ significantly.

7.3.1.5 Spleen

Early litter exposure had no significant effect on relative spleen weight at 35 days (Table 7.6); however, the effect was significant at 42 days of age (P = 0.0099, Table 7.6) with relative bursal weights of exposed chickens being 15.9 % greater than those unexposed. Challenge material had a significant effect on relative spleen weight at 35 (P = 0.02) and 42 (P = 0.001) days of age (Table 7.6) with relative spleen weights of litter/blood challenged chickens being 31.1 % and 20.6 % greater than buffer challenged chickens at each age respectively.

The interaction between the effects of early litter exposure and challenge material was non-significant at 35 days of age; however, significant at 42 days of age (P = 0.016) with unexposed buffer challenge chickens recording significantly less relative spleen mass than all other groups (Table 7.6, Figure 7.4). The interaction between the effects of early litter exposure and challenge material nested within allocated isolator were non-significant at both 35 and 42 days of age (Table 7.6).

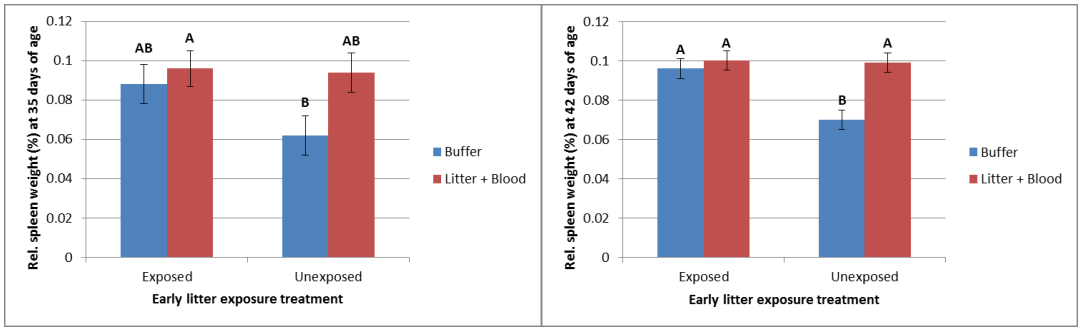

Figure 7.4 Experiment 1. Least-squares means (±SE) for relative spleen weights (% body weight) by early exposure to litter treatment and challenge material at 35 (left panel) and 42 (right panel) days of age. Columns not sharing a common letter differ significantly.

Table 7.6 Summary of analysis of bodyweight (BWt) and relative bursa of Fabricius and spleen weight data for experiment 1, presenting least squares means ± standard error and P-values showing the effect of each experimental factor/s. Figures with a different letter differ significantly.

Parameter/Treatment/Effect	**LSM±SEM and P values for different variables**		
	BWt (g)	**Rel. bursal wt (%)**	**Rel. spleen wt (%)**
35 days of age	1335±35	0.148±0.012	0.085±0.005
EARLY LITTER EXPOSURE	**P = 0.03**	P = 0.20	P = 0.22
Exposed	1266±45^{B}	0.162±0.017	0.0906±0.0063
Unexposed	1420±49^{A}	0.131±0.017	0.0790±0.0068
CHALLENGE MATERIAL	P = 0.17	P = 0.27	**P = 0.02**
Buffer	1389±48	0.1331±0.0173	0.0734±0.0066^{B}
Blood + Litter	1297±47	0.1599±0.0165	0.0962±0.0065^{A}
EARLY LITTER EXPOSURE * CHALLENGE MATERIAL	P = 0.82	P = 0.56	P = 0.20
ISOLATOR [EARLY LITTER EXPOSURE * CHALLENGE MATERIAL]	P = 0.14	P = 0.08	P = 0.11
PM OPERATOR	N/A[1]	P = 0.90	P = 0.83
42 days of age	1832±27	0.144±0.006	0.091±0.003
EARLY LITTER EXPOSURE	P = 0.13	**P = 0.01**	**P = 0.009**
Exposed	1788±39	0.160±0.009^{A}	0.0978±0.0035^{A}
Unexposed	1871±38	0.129±0.009^{B}	0.0844±0.0035^{B}
CHALLENGE MATERIAL	P = 0.75	P = 0.47	**P = 0.001**
Buffer	1820.9±38.1	0.1400±0.0085	0.0826±0.0035^{B}
Blood + Litter	1838.5±39.3	0.1489±0.0088	0.0996±0.0036^{A}
EARLY LITTER EXPOSURE * CHALLENGE MATERIAL	P = 0.60	P = 0.63	**P = 0.016**
Exposed/Buffer			0.095±0.005^{A}
Exposed/Blood+Litter			0.100±0.005^{A}
Unexposed/Buffer			0.070±0.005^{B}
Unexposed/Blood+Litter			0.099±0.005^{A}
ISOLATOR [EARLY LITTER EXPOSURE * CHALLENGE MATERIAL]	P = 0.61	P = 0.89	P = 0.82
PM OPERATOR	N/A[1]	P = 0.76	P = 0.64

[1]One operator.

7.3.1.6 Serology

None of the samples that were tested were seropositive for AEV, IBDV or Reo. A number of samples were seropositive for FAV8 and CAV (Table 7.7). In the case of FAV8 a significant effect of early litter exposure on FAV8 seropositivity was found (P = 0.001) with significantly more samples from early litter exposed groups being seropositive compared to unexposed groups. Additionally the effect of early litter exposure and challenge material nested within isolator was non-significant showing that variation was not present at the isolator level

In the case of CAV a significant effect of early litter exposure on CAV seropositivity was found (P = 0.016) with significantly more chickens not exposed to litter being seropositive compared to litter exposed groups. This result is surprising and is due to none of the samples from isolator 7 being seropositive (Table 7.7). This CAV serology was repeated, and the same results were derived. The isolator 7 result is an outlier with respect to the result from the other litter unexposed and sham-challenged group (isolator 1) being all seropositive indicating vertical transmission of this agent and may indicate a problem with the samples from isolator 7. The effect of challenge material on CAV seropositivity was found to be significant with more blood and litter challenged chickens being seropositive compared to sham- challenged chickens. The interpretation of this statistic is similarly complicated by the unusual results derived from samples tested from chickens from isolator 7.

Table 7.7 Serological results (percentages positive) from experiment 1

Isolator ID	Litter exposure at placement (Y/N)	Challenge material	No. of samples	AEV	IBDV	FAV8	Reo	CAV
1	N	Buffer	5	0 %	0 %	20 %	0 %	100 %
2	N	Blood + contaminated litter	5	0 %	0 %	40 %	0 %	100 %
3	Y	Buffer	5	0 %	0 %	80 %	0 %	80 %
4	Y	Blood + contaminated litter	5	0 %	0 %	60 %	0 %	100 %
5	N	Buffer	5	0 %	0 %	20 %	0 %	80 %
6	N	Blood + contaminated litter	5	0 %	0 %	20 %	0 %	100 %
7	Y	Buffer	5	0 %	0 %	80 %	0 %	0 %
8	Y	Blood + contaminated litter	5	0 %	0 %	80 %	0 %	100 %
Mean positive titre (±SE)				n/a	n/a	1.066±0.107*	n/a	2877±121
Mean negative titre (±SE)				1.425±0.425	21.575±6.958	0.187±0.026*	40.8±7.2	999±0
Effect of litter exposure at placement (P =)				1	1	**0.001**	1	**0.016**
Effect of challenge material (P =)				1	1	1	1	**0.001**
Effect of early litter exposure and challenge material nested within isolator (P =)				1	1	0.90	1	0.23

*Results reported as OD reading as titre not available. AEV = avian encephalomyelitis virus, IBDV = infectious bursal disease virus, FAV8 = fowl adenovirus serotype 8, Reo = reovirus, CAV = chicken anaemia virus.

6.3.2 Experiment 2

7.3.2.1 Reproduction of the APS

The APS was not reproduced in any chicken throughout the course of this experiment.

7.3.2.2 Survival

There were not any mortalities in either the spleen or blood challenged groups and there was 7.5 % mortality (3/40 chickens) in buffer challenged chickens (Figure 7.5). The effect of challenge material on survivability was non-significant (P = 0.25).

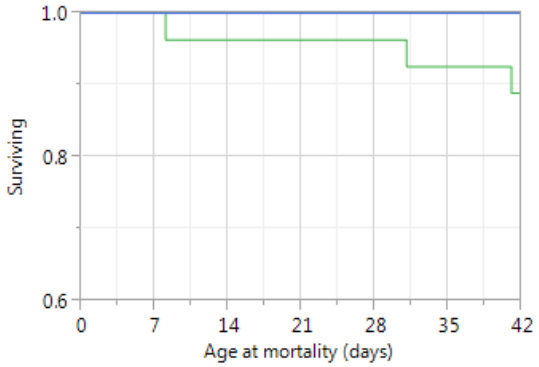

Figure 7.5 Survival patterns of chickens in experiment 2, showing mortality of three buffer-challenged chickens (green line).

7.3.2.3 Bodyweights

The mean bodyweight at 35 days of age was 1400±46 g and at 42 days of age 1843±32 g. The effect of challenge material on bodyweight was not significant at 35 or 42 days of age (Table 7.8, Figure 7.6).

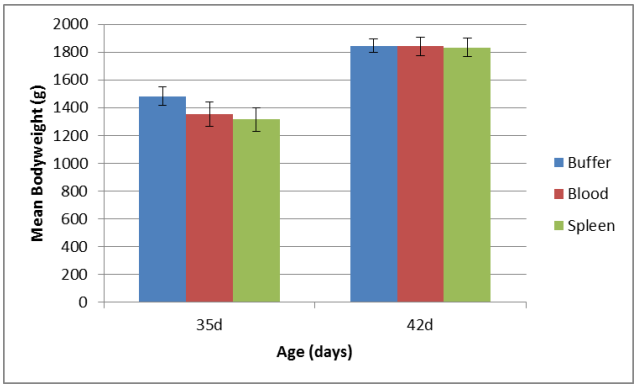

Figure 7.6 Experiment 2. Least squares means (±SE) for bodyweights by challenge material at 35 and 42 days of age. The effect of challenge material was not significant at either age.

7.3.2.4 Bursa of Fabricius

The effect of challenge material on relative bursal weight was not significant at 35 (P = 0.20) and 42 days of age (Table 7.8). The mean relative bursal weights at both ages are shown in Figure 7.7.

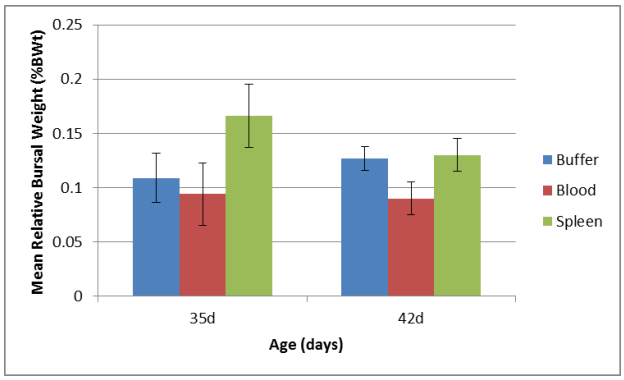

Figure 7.7 Least squares means (±SE) for relative bursa of Fabricius weights by challenge material at 35 and 42 days of age.

At the conclusion of the experiment the effect of PM operator on relative bursal weight was found to be significant (P = 0.02) and this was due to the mean relative bursal weights recorded by PM operator B being significantly lower than that from PM operator S but not that from P (Table 7.8).

7.3.2.5 Spleen

Challenge with blood, but not splenocytes, increased relative spleen weights by 35.2 % at day 35 and 39.5 % at day 42 (Table 7.8, Figure 7.8), most likely reflecting the presence of infectious agent/s in the blood challenge material inducing an immunological response of the spleen.

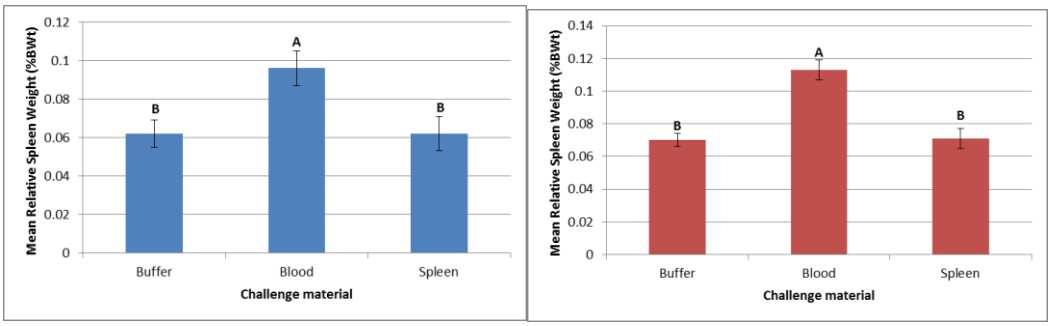

Figure 7.8 Experiment 2. Least-squares means (±SE) for relative spleen weights (% body weight) by challenge material at 35 (left panel) and 42 (right panel) days of age. In each graph columns not sharing a common letter differ significantly.

Table 7.8 Summary of analysis of bodyweight (BWt) and relative bursa of Fabricius and spleen weight data for experiment 2, presenting least squares means ± standard error and P-values showing the effect of each experimental factor/s. Figures with a different superscript differ significantly.

Parameter/Treatment/Effect	LSM±SEM and P values for different variables		
	BWt (g)	**Rel. bursal wt (%)**	**Rel. spleen wt (%)**
35 days of age	1400±46	0.121±0.016	0.071±0.005
CHALLENGE MATERIAL	P = 0.27	P = 0.20	**P = 0.01**
Buffer	1481±67	0.109±0.023	$0.062±0.007^{B}$
Blood (2^{nd} passage)	1354±86	0.094±0.029	$0.096±0.009^{A}$
Spleen (2^{nd} passage)	1314±86	0.166±0.029	$0.062±0.009^{B}$
PM OPERATOR	N/A[1]	P = 0.40	P = 0.10
42 days of age	1843±32	0.119±0.008	0.081±0.004
CHALLENGE MATERIAL	P = 0.99	P = 0.10	**P < 0.0001**
Buffer	1846.2±46.6	0.127±0.011	$0.070±0.004^{B}$
Blood (2^{nd} passage)	1840.5±65.9	0.090±0.015	$0.113±0.006^{A}$
Spleen (2^{nd} passage)	1834.1±65.9	0.130±0.015	$0.071±0.006^{B}$
PM OPERATOR	N/A[1]	**P = 0.02**	P = 0.43
B		$0.055±0.025^{B}$	
P		$0.110±0.014^{AB}$	
S		$0.130±0.009^{A}$	

[1]One operator.

7.3.2.6 Serology

None of the samples that were tested were seropositive to AEV, IBDV or Reo (Table 7.9). One sample (20%) from each sham-challenge isolator tested seropositive to FAV8 but none of the samples from blood or spleen challenged chickens were FAV8 seropositive (Table 7.8). The majority of sham-challenged chicken samples tested were seropositive to CAV; however, none of the samples tested from blood or spleen challenged chickens were seropositive to CAV. As a result, the effect of challenge material on CAV seropositivity was found to be significant ($P < 0.001$) (Table 7.9).

Table 7.9 Serological results (percentages positive) for experiment 2

Isolator ID	Challenge material	No. of samples	AEV	IBDV	FAV8	Reo	CAV
1	Buffer	5	0 %	0 %	20 %	0 %	100 %
2	Buffer	5	0 %	0 %	20 %	0 %	80 %
3	Blood	5	0 %	0 %	0 %	0 %	0 %
4	Spleen	5	0 %	0 %	0 %	0 %	0 %
Mean positive titre (±SE)			n/a	n/a	1.56±0.21*	n/a	2483±415
Mean negative titre (±SE)			1±0	24.75±11.41	0.14±0.02*	45.10±11.61	999±0
Effect of challenge material (P =)			1	1	0.53	1	**<0.001**

*Results reported as OD reading as titre not available. AEV = avian encephalomyelitis virus, IBDV = infectious bursal disease virus, FAV = fowl adenovirus serotype 8, Reo = reovirus, CAV = chicken anaemia virus.

7.3.3 Experiment 3

7.3.3.1 Reproduction of the APS

The APS was reproduced in one chicken in experiment 3 (bird 17, blood field B 200 µL). This chicken deceased overnight when it was at stage 3 of the APS so unfortunately a degree of PM degeneration of tissue had occurred by the time of tissue sampling. The clinical course in this chicken was identical to that reported for affected chickens in the experiment reported in Chapter 6 progressing through stages 1-3 of the APS. Figure 7.9 shows the affected chicken at stage 3 of the APS. The photograph, which was taken outside the isolator, shows a caudo-lateral view of the chicken in sternal recumbency with its head and neck on the isolator floor. This chicken, being in stage 3 of the APS, could not hold its head or neck off the floor and could not stand.

Figure 7.9 Bird 17 from experiment 3 demonstrating stage 3 of the APS.

7.3.3.2 Histopathology

Histological interpretations of fixed sections of brain and spinal cord from the affected chicken and two sham-challenged control chickens from the same isolator in experiment 3 are provided in Table 7.10. Figure 7.10 shows photographs of histological sections from the affected chicken which highlight some of the pathology as described in Table 7.10. In summary the affected chicken did not demonstrate inflammatory pathology in the brain and spinal cord as seen in cases described in the experiment reported in Chapter 6. However, Wallerian degeneration was present in the spinal cord from this chicken, similar to that observed in affected chickens as described in Chapter 6. Two in-contact sham treated chickens demonstrated similar degenerative pathology of the spinal cord.

Table 7.10 Summary of histopathological findings of brain and spinal cord sections from experiment 3 (interpretations provided by Dr. Rod Reece, SVDL)

Clinical state / Challenge material	**Bird ID / Age sampled**	**Histopathological lesions**
Affected/ Field B blood	Bird 17 / 43 days	**Brain:** significant PM degeneration with prominent tears, spaces and vacuoles, this could easily obscure an assessment of subtle changes. **Cervical cord:** several foci of clear wallerian degeneration on long section (LS), significant PM degradation in SS and transverse section (TS). **Thoracic cord:** multiple small foci of vacuolation, wallerian degeneration, swollen axons and macrophages in digestion chambers in LS. **Lumbar cord:** evidence of wallerian degeneration, but often obscured by autolytic changes.
Normal/ Buffer	Bird 39 / 47 days	**Brain:** vacuolation in cerebellar folia and within granular layer, equivocal perivascular oedema deep in the cerebellum. **Cervical cord:** several vacuolar spaces in TS. Chains of digestion chambers with occasional degenerate macrophages and swollen axons in LS. **Thoracic cord:** chains of digestion chambers with occasional degenerate macrophages and swollen axons in LS. **Lumbar cord:** chains of digestion chambers with occasional degenerate macrophages and swollen axons in LS.
Normal/ Buffer	Bird 40 / 47 days	**Brain:** small number of vacuoles in cerebellar white matter and granular layer. **Cervical cord:** small number of vacuolar spaces in lateral tracts devoid of axons on TS. Mild-moderate wallerian degeneration with digestion chambers, degenerate macrophages and pyknotic cells on LS. **Thoracic cord:** wallerian degeneration with digestion chambers and swollen axons, mitotic figures evident in oligodendroglial cells (LS). **Lumbar cord:** wallerian degeneration present but less pronounced than in the thoracic section examined.

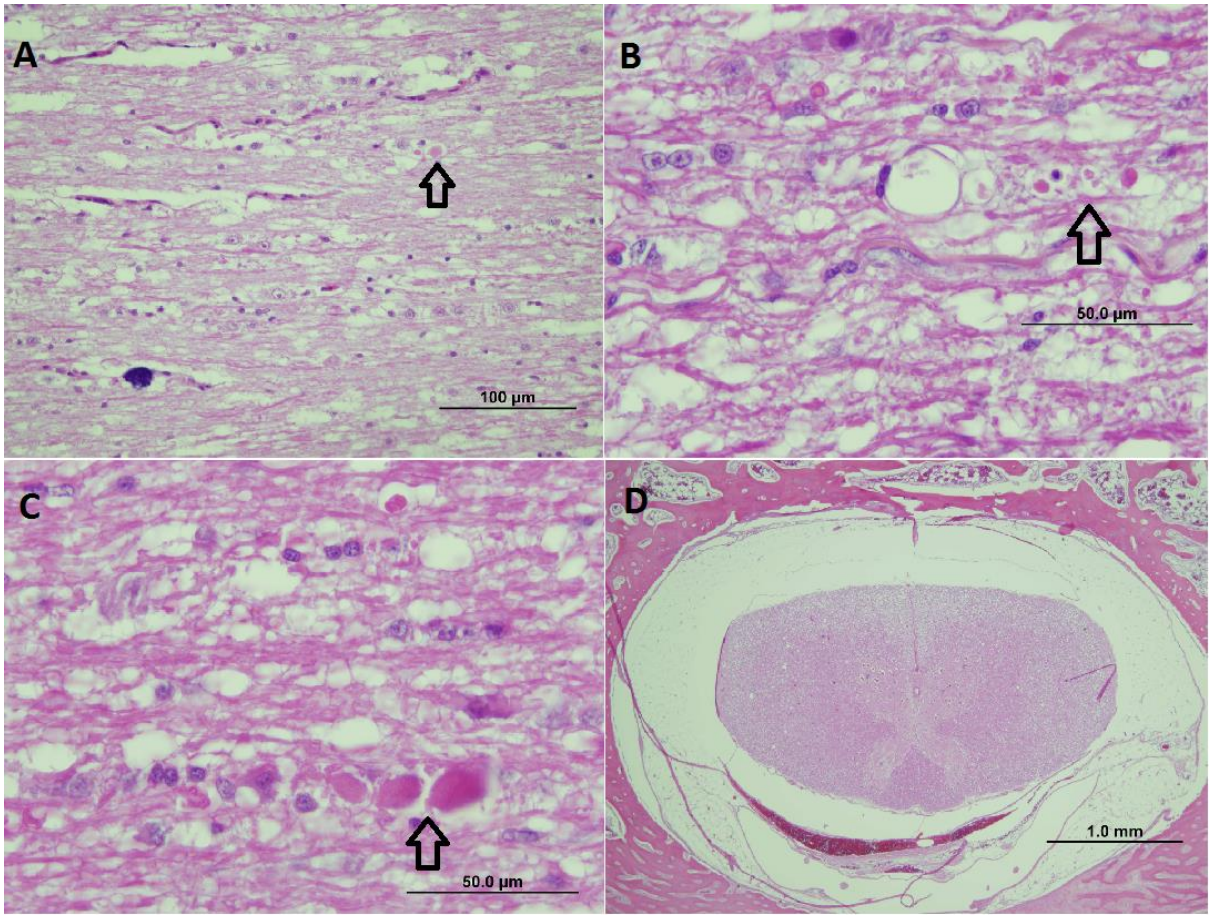

Figure 7.10 A: Thoracic spinal cord (LS) bird 17 showing large degenerate swollen axon (arrow). B. Cervical spinal cord (LS) bird 39 showing chains of digestion chambers with axonal debris (arrow). C: Cervical spinal cord (LS) bird 39 showing marked axonopathy (arrow) and axonal debris. D: Cervical spinal cord (TS) *in situ* from bird 40, showing marked vacuolation associated with the lateral white matter tracts. Photos from Dr. Rod Reece, SVDL.

7.3.3.3 Survival

Survival analysis revealed that both the effects of challenge material (P = 0.58) and challenge (P = 0.86) were non-significant. As is shown in Figure 7.11.B, mortality in the group of chickens dosed with 1,000 µL occurred closer to challenge as opposed to those chickens dosed with 200 µL. Figure 7.11.A highlights that three of the 10 chickens receiving the field blood B inoculum deceased within five days of challenge, two of which received the 1,000 µL dose. These two chickens demonstrated evidence of systemic bacterial infection on PM examination with severe fibrinous perihepatitis and pericarditis.

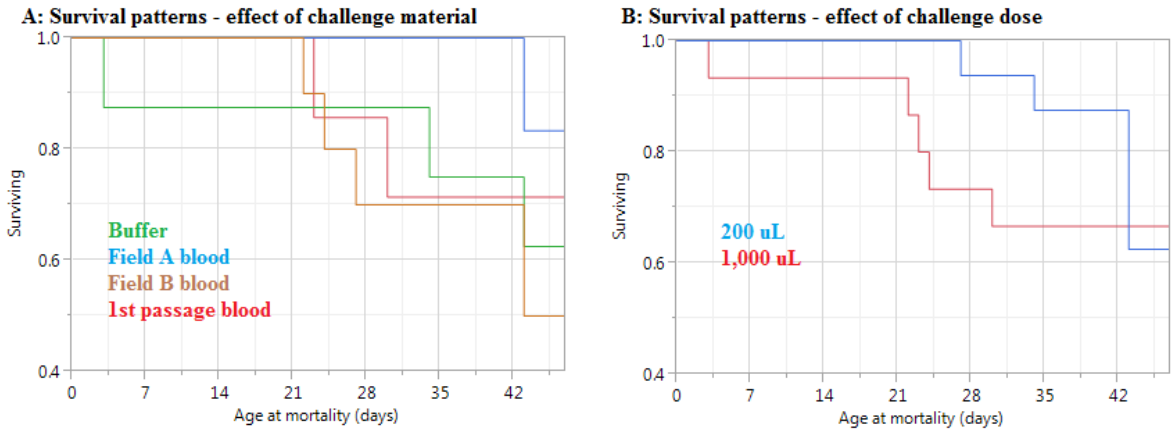

Figure 7.11 Survival patterns of chickens in experiment 3. A: The effect of challenge material was non-significant (P = 0.58). B: The effect of challenge dose was non-significant (P = 0.86).

7.3.3.4 Bodyweights

At 35 days of age the mean bodyweight was 1750±106 g which increased to 2451±95 g at 47 days of age. At 47 days of age bodyweight was 44.1 % greater for chickens administered 1,000 µL of challenge material compared to a dose of 200 µL (Table 7.11, Figure 7.12). With the absence of replication at the isolator level in this experiment it is impossible to draw inference as to whether this effect is truly dose dependent or due to other local effects specific to the isolator. At 47 days of age the effect of the challenge material type on bodyweight was non-significant (Table 7.11, Figure 7.12). At 47 days of age the interaction between the effects of challenge material and challenge dose on bodyweight was non-significant (Table 7.11).

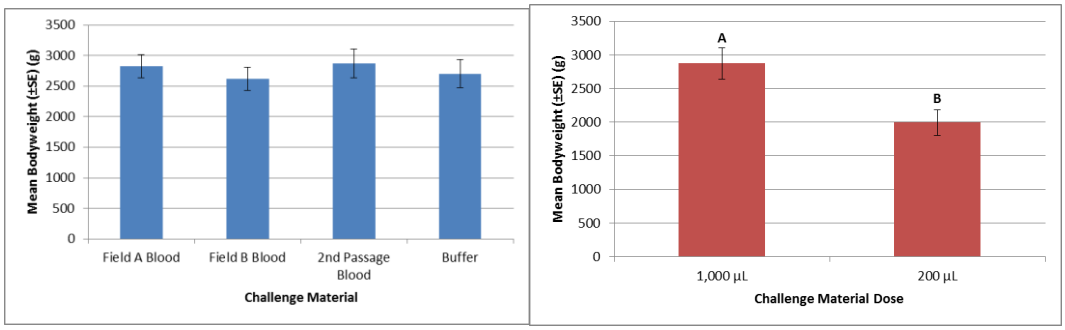

Figure 7.12 Experiment 3. Least squares means (±SE) for bodyweights at 47 days of age by challenge material (left panel) and challenge dose (right panel). Where indicated columns not sharing a common letter differ significantly.

7.3.3.5 Bursa of Fabricius

The effects of challenge material, challenge dose and the interaction between the effects challenge material and challenge dose on relative bursal weight were all non-significant (Figure 7.13, Table 7.11).

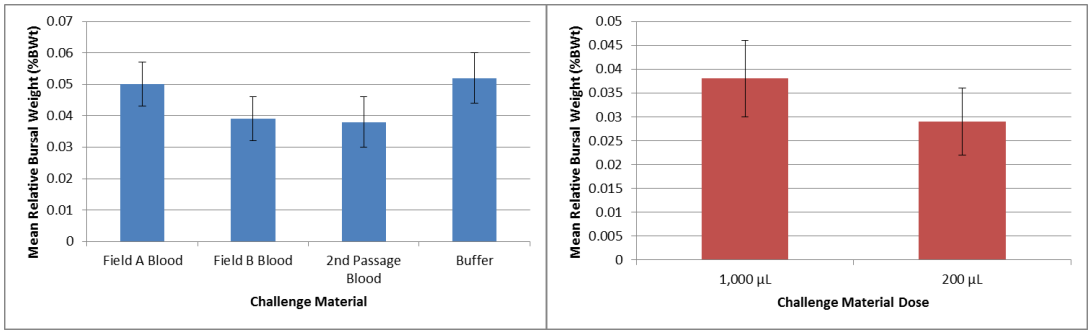

Figure 7.13 Experiment 3. Least squares means (±SE) for relative bursa of Fabricius weights by challenge material (left panel) and challenge material dose (right panel).

7.3.3.6 Spleen

The effects of challenge material, challenge dose and the interaction between the effects challenge material and challenge dose on relative spleen weight all non-significant (Table 7.11, Figure 7.14).

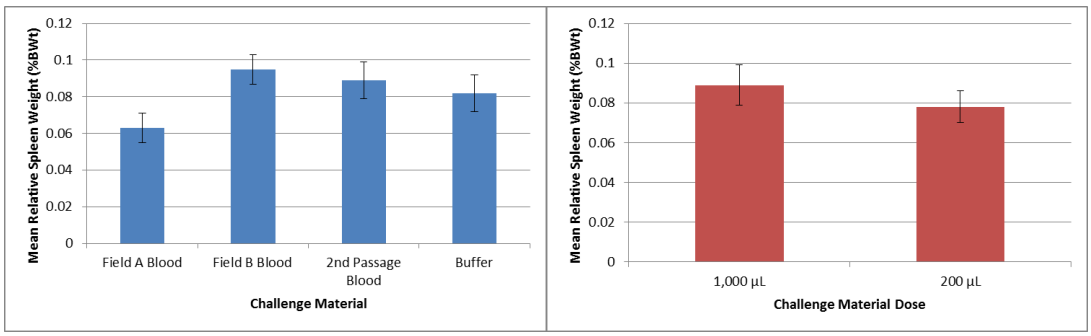

Figure 7.14 Experiment 3. Least-squares means (±SE) for relative spleen weights by challenge material (left panel) and challenge material dose (right panel).

Table 7.11 Summary of analysis of bodyweight (BWt) and relative bursa of Fabricius and spleen weight data for experiment 3, presenting least squares means ± standard error and P-values showing the effect of each experimental factor/s. Figures with a different superscript differ significantly.

Parameter/Treatment/Effect	**LSM±SEM and P values for different variables**		
	BWt (g)	**Rel. bursal wt (%)**	**Rel. spleen wt (%)**
35 days of age	1750±106	N/A[1]	N/A[1]
47 days of age	2451±95	0.0375±0.0029	0.0756±0.0038
CHALLENGE DOSE	**P = 0.01**	P = 0.46	P = 0.44
200 µL	1994±190[B]	0.0293±0.0068	0.0780±0.0084
1,000 µL	2873±233[A]	0.0375±0.0083	0.0885±0.0103
CHALLENGE MATERIAL	P = 0.82	P = 0.47	P = 0.09
Buffer	2703±233	0.0515±0.0083	0.082±0.010
Blood (field A)	2821±190	0.0500±0.0068	0.063±0.008
Blood (field B)	2621±190	0.0390±0.0068	0.095±0.008
Blood (2nd passage)	2873±233	0.0375±0.0083	0.089±0.010
DOSE * CHALLENGE MATERIAL	P = 0.47	P = 0.87	P = 0.81
PM OPERATOR	N/A[2]	N/A[2]	N/A[2]

[1]Not record, [2]One operator.

7.3.3.7 Serology

The data presented in Table 7.12 reports the levels of passive immunity provided to chicks at hatch and subsequent levels at 14 days of age specific to AEV, IBDV, FAV8, Reo and CAV. The reported percentages are those that are considered seropositive as per each kit's instructions. The results show a high level of maternal immunity in chickens at hatch against AEV, IBDV and FAV8 and moderate protection against CAV. This immunity remains high against IBDV through the first two weeks of life but wanes for AEV, FAV8 and CAV.

Table 7.12 Maternal antibody status of chickens used in experiment 3

Age		AEV	IBDV	FAV8	Reo	CAV
Day-old	Percentage positive (from 10 samples)	80 %	100 %	100 %	10 %	50 %
	Mean positive titre (±SE)	1673±253	8704±1318	2.34±0.33*	945±0	3173±1410
	Mean negative titre (±SE)	1±0	n/a	n/a	184±39	990±0
14 days	Percentage positive (from 10 samples)	20 %	100 %	40 %	0 %	20 %
	Mean positive titre (±SE)	748±352	4035±1203	1.69±0.58*	n/a	1591±697
	Mean negative titre (±SE)	59±27	n/a	0.18±0.03*	112±24	999±0

*Results reported as OD reading as titre not available. AEV = avian encephalomyelitis virus, IBDV = infectious bursal disease virus, FAV8 = fowl adenovirus serotype 8, Reo = reovirus, CAV = chicken anaemia virus.

The data presented in Table 7.13 reports the percentage of chickens which were seropositive at 47 days of age in each challenge material /challenge dose combination in relation to AEV, IBDV, FAV8, Reo and CAV. This testing failed to indicate any evidence of AEV, IBDV and FAV8 infection. One sham-challenged chicken was found to be seropositive for Reo and there was moderate levels of seropositivity to CAV through treatment groups. Challenge material and dose both had no significant effect on serological results for all of the tests reported.

Table 7.13 Serological results (percentages positive) for experiment 3 at 47 days of age

Challenge material	Dose	No. of samples	AEV	IBDV	FAV8	Reo	CAV
Buffer	200 µL	3	0 %	0 %	0 %	33 %	33 %
Field A Blood	200 µL	2	0 %	0 %	0 %	0 %	50 %
Field B Blood	200 µL	3	0 %	0 %	0 %	0 %	0 %
2nd Passage Blood	200 µL	3	0 %	0 %	0 %	0 %	0 %
Mean positive titre (±SE)			n/a	n/a	n/a	477±0	2973±1879
Mean negative titre (±SE)			32±7	27±12	0.09±0.01*	53±13	999±0
Buffer	1,000 µL	2	0 %	0 %	0 %	0 %	0 %
Field A Blood	1,000 µL	3	0 %	0 %	0 %	0 %	67 %
Field B Blood	1,000 µL	3	0 %	0 %	0 %	0 %	33 %
2nd Passage Blood	1,000 µL	2	0 %	0 %	0 %	0 %	50 %
Mean positive titre (±SE)			n/a	n/a	n/a	n/a	4365±446
Mean negative titre (±SE)			30±6	49±17	0.13±0.01*	34±9	999±0
Effect of challenge material (P =)			1	1	1	0.99	0.50
Effect of challenge dose (P =)			1	1	1	0.77	0.37

*Results reported as OD reading as titre not available. AEV = avian encephalomyelitis virus, IBDV = infectious bursal disease virus, FAV = fowl adenovirus serotype 8, Reo = reovirus, CAV = chicken anaemia virus.

7.4 Discussion

The challenge models used in the three described experiments were based on the ability to reproduce the APS in broiler chickens when challenged at 21 days of age using blood, spleen or litter as challenge materials (Chapter 6). Blood and litter were considered the two best challenge materials due to the convenience in collection and storage of each compared to the lengthy process required for the extraction and cryopreservation of spleen cells (Chapter 3). All field harvested blood and litter were different to that used in the experiment described in Chapter 6 as residual material from that experiment was not available.

In experiments 1 and 2 (13-C-REP2 and 13-C-REP3) chickens were reared on litter flooring which differed to the experiment described in Chapter 6 in which the APS was successfully reproduced. The reason for the modification of flooring was in attempt to improve chicken welfare by attempting to reduce the incidence of lameness and provide environmental enrichment with litter. Complications associated with this modification were that the design of the feeders in the isolators did not allow them to be raised which meant that feeder outlets were being regularly filled with litter. This complication likely restricted feed intake. Additionally the inclusion of litter trays into the isolators likely restricted air flow between the air inlet and outlet thus reducing air quality. Finally, the litter became significantly caked in most isolators by the end of each experiment, despite regular attempts at turning it, which may have actually increased the incidence of lameness via damage to the foot pads and hocks.

For 13-C-REP2, the hypothesis that chickens exposed to contaminated shed litter at placement and challenged at 21 days of age would show a significantly reduced prevalence of the APS compared to chickens that did not receive such exposure could not be tested due to the inability to reproduce the APS. There were several identifiable differences in this experiment to that described in Chapter 6 (13-C-REP1) which may explain why the APS could not be reproduced. Firstly, challenge material used in each was different in origin thus the challenge material used for this experiment may have not actually contained the infectious agent/s or contained non-pathogenic doses of the agent/s.

Secondly, the mean bodyweight at 35 days of age was 1335±35 g, which was lower than that achieved in 13-C-REP1 in which the mean male bodyweight at 35 days of age was 2045±79 g and the Ross 308 guidelines for male bodyweight at 35 days (2250 g) (Aviagen, 2012). Thus, growth rates were lower in this experiment and reduced growth rates may explain the absence of the APS, given field observations that the risk of the APS is greater in heavier flocks (Chapter 5).

Figure 7.15 plots mean bodyweights by age achieved in each of the three experiments reported in this chapter along with those of males from 13-C-REP1 and the guidelines for males of the Ross 308 breed (Aviagen, 2012). The data between each measurement date for each experiment is interpolated. It highlights that at the points of measurement, bodyweights were lowest in 13-C-REP2 and 13-C-REP3 which failed to reproduce the APS. 13-C-REP4 achieved higher bodyweights but did not match those achieved in 13-C-REP1 or the Ross 308 guidelines. Problems associated with isolator flooring design may have affected feed availability and/or air quality and may explain the reduced growth rates achieved in experiments 1 and 2.

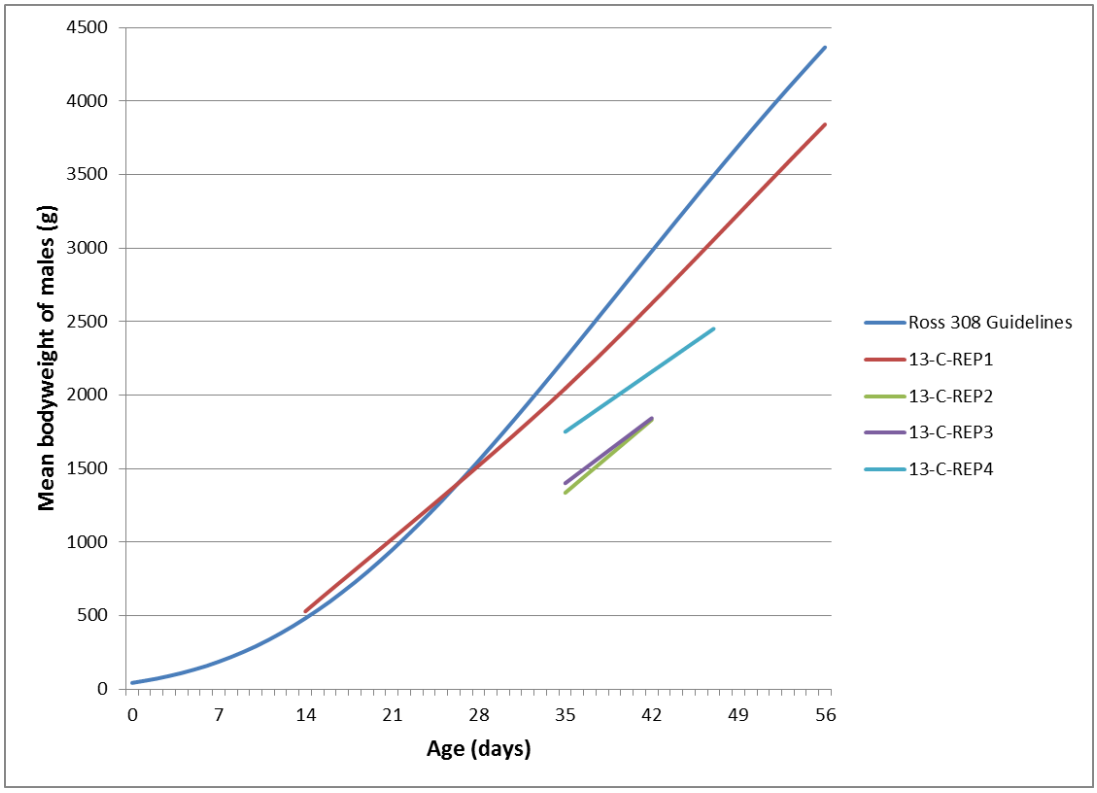

Figure 7.15 Mean bodyweights achieved in experiments 1-3 in comparison to the Ross 308 guidelines (Aviagen, 2012) and results from the experiment reported in Chapter 6 (13-C-REP1).

Finally, parent flocks for the chickens sourced for the experiments were different to that for 13-C-REP1 (Table 7.14). Parent flocks may play a role in the presence or absence of the APS in a flock of chickens in two ways. Firstly whether the MAb provided to the chicks is protective and for how long it remains protective may be a factor. In the experiment reported in Chapter 6 chickens challenged at day old did not develop the APS whereas chickens challenged at 21 days of age were susceptible. Secondly, parent flocks may play a role if there was vertical transmission of the putative agent/s.

Table 7.14 Parent flock to the chickens used in experiments

Experiment	Parent flock identification
13-C-REP1 (Chapter 6)	B
13-C-REP2	C
13-C-REP3	C
13-C-REP4	A

In 13-C-REP2 early litter exposure did not have a significant effect on final bodyweight; however, relative spleen and bursa of Fabricius weights were significantly heavier in exposed compared to unexposed chickens. These results suggest that there were infectious agents in the litter which provided immunological challenge to the chickens but did not affect performance in relation to bodyweight. In this experiment challenge material did not affect body or relative bursal weights but resulted in significantly increased relative spleen weights, in line with the expected immunological response of the spleen to infectious agents present in challenge material. It is perhaps a surprising finding that challenge material did not significantly increase relative bursa of Fabricius weight given that early litter exposure did. Early litter exposure is likely to affect gut microbiota, which indirectly provides immune stimulation.

For 13-C-REP3, due to the inability to reproduce the APS the prediction that 1st passage whole blood and splenocytes harvested from chickens displaying APS from the experiment 13-C-REP1 would induce APS in broilers in its second chicken passage could not be tested. Similar differences existed as described for 13-C-REP2 that may explain the absence of the APS in this experiment. The absence or reduced concentration of the putative infective agent/s in the first chicken passage challenge material could also explain the failure to reproduce the APS in this experiment. This could result if there was a difference in time point of the disease at harvesting of infective material which could affect the dose of the putative agent/s in the material as compared to the challenge material used for the experiment 13-C-REP1.

Similar to 13-C-REP2 relative spleen weights were significantly heavier in challenged chickens compared to sham-challenged chickens and this difference was also detected at 35 days of age in 13-C-REP3. Mean relative spleen weight was significantly greater in blood challenged chickens compared to spleen- and sham-challenged chickens suggesting the blood challenge material induced a greater immunological response in the chickens.

The third experiment (13-C-REP4) was designed to test the infectivity of three sets of separately harvested infective materials and to test the prediction that the prevalence of APS induced in chickens exposed to potentially infective material would be dose dependant. The isolator design

did not include litter trays in this experiment due to the problems encountered with feeders being blocked with litter in 13-C-REP2 and 13-C-REP3. The APS appeared to be reproduced in one chicken which was inoculated with the field blood B inoculum. This bird clinically progressed through the APS as described in Chapter 6. Brain histopathological findings were not found to be consistent with that reported in Chapter 6; however, PM degenerative changes could have disguised any subtle inflammatory brain lesions if present. Degenerative changes were evident in the spinal cord of this chicken as previously reported; however, these changes were also evident in two in-contact sham challenged broilers. This could be a result of horizontal transmission of an infective agent or agents from inoculated chickens inducing the neurological changes evident but not inducing clinical disease. Alternatively, Wallerian degenerative changes may always be present in broiler chickens at this age. However, there does not appear to be any literature supporting this idea.

From 13-C-REP4 it is not possible to determine any dose effect on the development of APS. In relation to the field blood B inoculum, three of the five chickens receiving 1,000 µL of this inoculum deceased within five days of challenge. This period of time is within the period from challenge to development of the APS as reported for 13-C-REP1. Hence these early mortalities severely limited the chances of observing the APS in this group which is an obvious fault in experimental design due to facility limitations at the time.

As shown in Figure 7.15, higher bodyweights were achieved in 13-C-REP4 compared to 13-C-REP2 and 13-C-REP3; however, not as high as those achieved for 13-C-REP1. At 35 days the mean bodyweight of chickens was 1750±106 g which was lower than the 2045±79 g achieved in 13-C-REP1. Thus this could explain the low incidence of APS in 13-C-REP4 compared to 13—REP1.

Serological results derived from experiments reported in this chapter suggest the presence of FAV8 and CAV to varying degrees consistent with transmission of the agents both vertically and via challenge material and these results were consistent with those reported in Chapter 6. In 13-C-REP2 it was evident that a level of vertical transmission of FAV8 was present; however, early exposure to litter increased exposure to the virus. From 13-C-REP3, the majority of sham-challenged chicken samples tested were seropositive to CAV; however, none of the samples tested from blood or spleen challenged chickens were seropositive to CAV. This was an interesting finding and possibly reflects that there was sufficient passive transfer of immunity in the blood and spleen challenge material to inhibit further CAV replication from challenge.

The most obvious difference between the serological profiles from the experiments reported in this chapter compared to those from 13-C-REP1 reported in Chapter 6 was the lack of seropositivity to AEV in the former. This is an interesting finding but not linked to the difficulty in reproducing the APS given the diagnostics reported in Chapter 6 which ruled out AEV as a causative agent of the APS.

7.5 Conclusion

The inability to reproduce the APS in both 13-C-REP2 and 13-C-REP3 and with two of the three challenge materials used 13-C-REP4 highlights the need for work to focus on the development of a more effective disease reproduction model for further studies on the APS. Efforts should focus on achieving growth rates nearest to the Ross 308 guidelines in order to increase the chances of reproducing the APS. Given the apparent ability to reproduce the APS using the field blood B inoculum it should be used as challenge material in future experiments. Further histopathological evaluations should focus on determining whether Wallerian degenerative changes are present in true sham-challenged controls rather than in-contact birds, which will assist in determining whether this histopathological finding is associated with the APS.

Chapter 8: The effect of immunosuppression on the development of an acute paralysis syndrome of broiler chickens

8.1 Introduction

This experiment follows from the results obtained from the experiments reported in Chapters 6 and 7. From the experiment reported in Chapter 6 it was evident that the acute paralysis syndrome (APS) is experimentally reproducible by challenging Ross 308 broiler chickens with spleen cells, whole blood or contaminated litter harvested from field cases of the APS. However, the experiments reported in Chapter 7 demonstrated that the APS cannot be reliably reproduced in an experimental setting and there are a number of reasons considered for this including, variations in growth rates between experiments, variations in challenge material used between experiments and variations in batches of chickens, as previously discussed. In order to continue investigations into the causation of the APS a reliable experimental reproduction model needs to be developed.

It is a recognised concept that the immunocompromised animal is at increased risk of disease caused by infectious agents. The ability to experimentally reproduce the APS in the experiment reported in Chapter 6 suggests that there is an infectious component to the APS. In order to develop a more reliable experimental reproduction model for the APS, it is proposed that by immunosuppressing broiler chickens, their susceptibility to the APS would be increased. Treating chickens with dexamethasone (DEX) or cyclophosphamide (CY) are documented methods of experimentally inducing immunosuppression.

DEX acts to suppress cell-mediated immunity by inhibiting genes which code for a range of cytokines, the most important being interleukin-2, required for T-cell proliferation (Leung & Bloom, 2003). Isobe & Lillehoj (1993) found that treatment of chickens with DEX prior to inoculation with *Eimeria mivati* oocysts, resulted in suppressed T-cell immunity and increased susceptibility to *E. mivati* and that the intramuscular injection of DEX was more effective at inducing immunosuppression compared to oral administration. Conversely, Martin *et al.* (2011), found that administration of 2 mg/kg DEX by intramuscular injection at the day of inoculation with *Enterococcus cecorum* did not increase susceptibility to *E. cecorum* clinical disease. Huff *et al.* (1998) effectively increased susceptibility to *Escherichia coli* in turkey poults administered 2 mg/kg DEX into the thigh muscle on 3 alternating days from 5 weeks of age prior to inoculation with *E. coli.*

CY administration to chickens has a prolonged suppressive effect on the humoral (B-cell) immune system of chickens (Lerman & Weidanz, 1970). Additionally, Sharma & Lee (1977) found that CY treatment of chickens had a short term suppressive effect on the T-cell mediated immune system; however, much shorter than are the suppressive effects on the B-cell mediated system. CY is cytotoxic and induces cell apoptosis by causing the covalent linkage of alkyl groups to DNA in cells with low concentrations of aldehyde dehydrogenase (Hall & Tilby, 1992), such as lymphocytes. Westbury & Sinkovic (1978b) found chickens treated with CY for four consecutive days from hatch (4 mg in 0.1 mL injected IA) and subsequently inoculated with avian encephalomyelitis (AE) virus at various ages based on treatment group were more susceptible to clinical disease, and CY treatment removed the normal age-associated resistance to this disease.

The experiment reported in this chapter was designed to immunosuppress broiler chickens prior to challenging them with spleen cells, blood or contaminated litter harvested from field cases of the APS. Immunosuppression was induced by treatment of chickens with either DEX or CY. Challenge material was harvested from two cases of the APS. The first batch of material was the same as that which was used in experiment 3 reported in Chapter 7 in which the APS was reproduced in one chicken and the second batch of material was harvested from a more recent case of the APS. The experiment was conducted in the University of New England (UNE) poultry isolator facility using Ross 308 broiler chickens sourced from the same hatchery as used for the experiments reported in Chapters 6 and 7. Litter floor trays were installed into isolators and rice hulls used as litter material in order to match the litter used in commercial broiler sheds experiencing the APS.

From the experiment reported in Chapter 7, Wallerian degeneration was observed in spinal cord sections from both challenged and in-contact sham-challenged broiler chickens. It was not possible to determine whether the degenerative pathology observed in sham-challenged chickens was incidental or in fact resultant from horizontal transmission of an infectious agent/s. The design of the experiment reported in this chapter allowed for spinal cord histology to be examined from sham-challenged chickens which were not in contact with challenged chickens in order to assess for the presence of Wallerian degeneration.

8.2 Materials and Methods

8.2.1 Experimental design and application of treatments

This experiment (14-C-REP5) was approved by the University of New England (UNE) Animal Ethics Committee (AEC 14-015), commenced 17th March 2014 and was terminated in 7th May 2014. The experiment used a 2 x 6 x 4 factorial design (Table 8.1) with two replicates, utilising 480 unsexed broiler chickens. A total of 20 chickens were placed into each of 24 isolators at the commencement of the experiment. The three experimental factors were:

- Chicken batch: two batches were used with each batch hatched on consecutive days in the same hatchery and each batch from separate parent flocks.
- Challenge material: six challenge materials were used for challenge of chickens at 21 days of age and included sterile phosphate buffered saline (PBS) as a sham-challenge, blood collected from affected chickens from field outbreak B which was the same material that induced the APS in 13-C-REP4 (Blood B), litter collected from an affected shed from field outbreak B (Litter B), spleen cells harvested from affected chickens from field outbreak C (Spleen C), blood collected from affected chickens from field outbreak C (Blood C) and litter collected from an affected shed from field outbreak C (Litter C).
- Immunosuppressive treatment: four treatments were used including, CY, DEX, PBS administered via the same challenge method as CY (PBS1) as a CY-sham-treatment and PBS administered via the same challenge method as DEX (PBS2) as a DEX-sham-treatment.

Each batch of chickens was transported to UNE via air freight from the hatchery on the day of hatch. The first batch of chickens was placed into allocated isolators on day 0 (Expt d0) of the experiment and the second batch was placed on day 1 (Expt d1) of the experiment. The experiment was terminated on day 51 (Expt d51) when batch 1 were 51 days of age and batch 2 were 50 days of age.

Chickens were toe web marked based on their allocated treatment prior to placement in the isolators. CY and PBS1 treatment chickens were administered 0.1 mL of CY (40 mg/mL) (Endoxan 500mg, Baxter Healthcare Pty Ltd., Toongabbie NSW, Lot: 3F752B) or 0.1 mL PBS respectively by IA injection once daily for four days commencing from the day of placement. DEX and PBS2 treatment chickens were administered 0.15 mL of DEX sodium phosphate (5 mg/mL) (Ilium Dexapent, Troy Laboratories Pty Ltd., Glendenning NSW, Batch: 131120) or

0.15 mL of PBS respectively by intramuscular injection into alternating thigh muscles once daily on the 18th, 19th, 20th and 21st day of age.

Blood and spleen challenge materials were cryopreserved at UNE (Chapter 3). Allocated chickens were inoculated with 200 µL of either inoculum by intra-abdominal (IA) injection on the 21st day of age. Litter collected from field outbreaks was stored at -20°C at UNE until ready for use. In allocated isolators, approximately 1 kg of contaminated litter was spread evenly and mixed through the rice hulls and sprinkled through the openings to feeders on the 21st day of age.

Table 8.1 Experimental design

Isolator ID	Number of chickens	Chicken batch	Challenge Material	Treatment (Number of chickens)
1	20	1	PBS	PBS1(4), CY(6), PBS2(4), DEX(6)
13	20	1	PBS	PBS1(4), CY(6), PBS2(4), DEX(6)
2	20	1	Spleen C	PBS1(4), CY(6), PBS2(4), DEX(6)
14	20	1	Spleen C	PBS1(4), CY(6), PBS2(4), DEX(6)
3	20	1	Blood C	PBS1(4), CY(6), PBS2(4), DEX(6)
15	20	1	Blood C	PBS1(4), CY(6), PBS2(4), DEX(6)
4	20	1	Litter C	PBS1(4), CY(6), PBS2(4), DEX(6)
16	20	1	Litter C	PBS1(4), CY(6), PBS2(4), DEX(6)
5	20	1	Blood B	PBS1(4), CY(6), PBS2(4), DEX(6)
17	20	1	Blood B	PBS1(4), CY(6), PBS2(4), DEX(6)
6	20	1	Litter B	PBS1(4), CY(6), PBS2(4), DEX(6)
18	20	1	Litter B	PBS1(4), CY(6), PBS2(4), DEX(6)
7	20	2	PBS	PBS1(4), CY(6), PBS2(4), DEX(6)
19	20	2	PBS	PBS1(4), CY(6), PBS2(4), DEX(6)
8	20	2	Spleen C	PBS1(4), CY(6), PBS2(4), DEX(6)
20	20	2	Spleen C	PBS1(4), CY(6), PBS2(4), DEX(6)
9	20	2	Blood C	PBS1(4), CY(6), PBS2(4), DEX(6)
21	20	2	Blood C	PBS1(4), CY(6), PBS2(4), DEX(6)
10	20	2	Litter C	PBS1(4), CY(6), PBS2(4), DEX(6)
22	20	2	Litter C	PBS1(4), CY(6), PBS2(4), DEX(6)
11	20	2	Blood B	PBS1(4), CY(6), PBS2(4), DEX(6)
23	20	2	Blood B	PBS1(4), CY(6), PBS2(4), DEX(6)
12	20	2	Litter B	PBS1(4), CY(6), PBS2(4), DEX(6)
24	20	2	Litter B	PBS1(4), CY(6), PBS2(4), DEX(6)

CY = cyclophosphamide, DEX = dexamethasone, PBS = phosphate buffered saline.

8.2.2 Chicken maintenance, measurements and sample collection

The feed loaded into each isolator was locally sourced and consisted of commercially available starter and grower rations. At the placement of each batch an additional 10 chickens were sacrificed for blood collection (terminal) and subsequent serum extraction in order to determine maternal antibody levels for a selection of infectious agents. At placement a mean chicken weight for each batch was calculated.

On the 21st day of the experiment (Expt d21) blood was collected from a cutaneous ulnar vein from a total of 12 DEX treated and four PBS2 treated chickens. Blood was collected into plain serum tubes (BD Vacutainer®, Ref: 366668) from which serum was subsequently extracted and

stored (-20°C) for later blood glucose measurements in order to determine the efficacy of DEX administration.

On the 36th day of the experiment (Expt d36) the number of chickens in each isolator was reduced to 12, leaving a total of 4 x CY, 4 x DEX, 2 x PBS1 and 2 x PBS2 treated chickens in each isolator where numbers remaining permitted. On Expt d51 all surviving chickens were euthanased via cervical dislocation. On Expt d36 and Expt d51 bodyweight measurements were recorded from each euthanased chicken prior to a post-mortem (PM) examination. From the PM examination spleen and bursal weights were recorded. Additionally on Expt d51 prior to euthanasia, blood was collected from a total of four chickens per isolator (one per treatment allocation) into plain serum tubes (serum was subsequently extracted and stored at -20°C) and EDTA tubes (BD Vacutainer®, Ref: 367839) for subsequent serological and haematological analyses.

Sections of brain and spinal cord were stored fixed in 10% neutral buffered formalin from a selection of challenged and sham-challenged chickens on Expt d51. Various tissues were collected for fixed and fresh (-20°C) storage from deceased and euthanased chickens throughout the experiment. Chickens were visually inspected twice daily.

8.2.3 Laboratory techniques

8.2.3.1 Histopathology

Fixed tissue sections were sent to the State Veterinary Diagnostic Laboratory (SVDL) for histological evaluation by avian pathologist Dr. Rod Reece. This service was provided by the SVDL at commercial rates.

8.2.3.2 Serology

Serological testing on serum extracted and stored from a selection of chickens in the experiment was performed by Birling Avian Laboratories (Bringelly, NSW). Tests were conducted using commercially available ELISA kits for AE virus (AEV), infectious bursal disease virus (IBDV), fowl adenovirus serotype 8 (FAV8), reovirus (Reo) and CAV. These diseases were selected because there were varying levels of seropositivity to each in the experiments reported in Chapter 6. The details specific to each test kit used are provided in Table 8.2.

Table 8.2 ELISA kit details for serological tests performed at Birling Avian Laboratories

Disease/Agent	Test, Source
AEV	ELISA, IDEXX Laboratories, Batch: 99-09259 LJ345, Expiry: 30/03/2015
IBDV	ELISA, IDEXX Laboratories, Batch: 99-09260 AK668, Expiry: 13/06/2015
FAV8	ELISA, Trop Bio Pty Ltd., Batch: IBHMW02, Expiry: 02/2014
Reo	ELISA, IDEXX Laboratories, Batch: 99-09264 DJ344, Expiry: 26/03/2014
CAV	ELISA, IDEXX Laboratories, Batch: 99-08702 EJ244, Expiry: 29/09/2014

AEV = avian encephalomyelitis virus, IBDV = infectious bursal disease virus, FAV8 = fowl adenovirus serotype 8, Reo = reovirus, CAV = chicken anaemia virus.

8.2.3.3 Molecular diagnostic techniques

Detailed descriptions of DNA extraction procedures and PCR protocols are provided in Chapter 3.

PCR for the detection of FAV8

DNA amplification reactions were set up (Chapter 3) utilising the primer set (*F3/B3*) reported by Renz *et al.* (2015) which flanks a 229 bp region within the *ORF33a* gene in the FAV8 genome in order to test for the presence of FAV8 in liver samples from a selection of chickens from the experiment. DNA extracted from the liver of an SPF chicken challenged with FAV8 from another experiment at UNE was used as a positive control for this assay.

PCR and qPCR for the detection of CAV

DNA amplification reactions were set up (Chapter 3) utilising a primer set which flanks a 180 bp region within the ORF3 gene in the CAV genome (Renz, unpublished data) in order to test for the presence of CAV in liver samples from a selection of chickens from the experiment. DNA extracted from the liver of an SPF chicken challenged with CAV from another experiment at UNE was used as a positive control for this assay. Quantitative PCR (qPCR) (Zhang *et al.*, 2009) was used to test for the presence of CAV in liver samples from a selection of chickens from the experiment.

8.2.3.4 Haematology

Haematological analyses, specifically absolute and relative white blood cell counts, were performed using a Cell-Dyn 3700 (Abbott Laboratories, Illinois, USA) at the FD McMaster Laboratory, Commonwealth Scientific and Industrial Research Organisation (CSIRO), 'Chiswick', Armidale NSW. Blood samples collected into EDTA tubes were delivered to the laboratory within 4 hours of collection and the service was performed by the CSIRO at commercial rates.

8.2.3.5 Serum glucose measurement

Stored serum samples (-20°C) sampled on Expt d21 were sent to Specialist Diagnostic Services Pty Ltd., North Ryde NSW, for serum glucose measurement. This service was provided at commercial rates.

8.2.4 Statistical analysis

Statistical analysis of experimental data was conducted using the software package JMP 10 (SAS Institute Inc., 2012). A significance level of $P < 0.05$ was accepted for all statistical tests and in all cases the individual chicken was the experimental unit. Time to event analyses, quantifying the time taken for birds to die or require euthanasia (due to the APS) following enrolment into the study (in days), were described using Kaplan-Meier survival curves. All chickens that were euthanased on Expt d36 for scheduled measurements and sampling were excluded from such analyses.

Bodyweight measurements on Expt d 36 and 51 for batch 2 chickens were corrected for age to allow for comparison with batch 1 chickens. This was achieved by adding the average daily weight gain (derived from the average batch 2 chicken weight at placement) for each chicken on Expt d 36 and 51 to each weight respectively. This allowed the true bodyweights of batch 1 chickens at 36 and 51 days of age to be compared with the corrected bodyweights of batch 2 chickens on Expt d36 and 51. Relative immune organ weights (bursa of Fabricius and spleen) were expressed as a percentage of bodyweight (relative organ weight = [(organ weight/bodyweight) x100]. For continuous data including bodyweight and relative immune organ weights, analyses were conducted using a general linear model fitting the effects of immunosuppression, challenge material, chicken batch, sex, two-way interactions between each of these factors, and post-mortem (PM) operator, each where applicable. Additionally the model included the effect of isolator nested within chicken batch and challenge material in order to test the effect of isolator. From such analyses, least squares means (LSM) and standard errors (SE) are presented and Tukey's HSD test was used to determine the significance of differences between more than two means. Additionally a general linear model was used to test the effect of immunosuppressive treatment on absolute and relative white blood cell counts. CAV load / mg of liver tissue was $\log_{10}$ transformed [$\log_{10}$ (x + 1)] prior to fitting the effects of treatment, challenge material and challenge material and batch nested within isolator in a general linear model.

Analysis of discrete serological data (positive or negative) was performed using a general linear model with a binomial link function (logistic) fitted for the effects of treatment, challenge, sex, batch and batch and challenge material nested within allocated isolator. A one-way ANOVA

model testing the effect of chicken batch on antibody titre was used to analyse positive titres from sera sampled at placement. In order to test the efficacy of DEX administration, serum glucose concentration (mmol/L) was assessed using a one-way ANOVA model fitting the effect of immunosuppressive treatment.

8.3 Results

8.3.1 Survivability

The clinical signs of the APS were not reproduced in this experiment. Survival analyses were grouped by chicken batch, sex, immunosuppressive treatment and challenge material. The Kaplan-Meier survival curves for each grouping are shown in Figure 8.1. The effects of batch ($P = 0.39$), sex ($P = 0.63$) and challenge material ($P = 0.89$) on chicken survival were non-significant. Immunosuppressive treatment had a significant effect on survivability ($P < 0.001$) and as is shown in Figure 8.1.C, the survivability of the CY treatment group was significantly reduced. The PBS1 treatment group had reduced survivability compared to the DEX and PBS2 groups, with most of the mortalities from the PBS1 group being within the first 10 days of life, which was most likely due the process of four daily injections in these chickens during the first four days of life. Hence the immunosuppressive treatment handling procedure needs to be taken into account when interpreting the survival curve for the CY treatment group. Thus mortalities after 13 days of age (Figure 8.1.C) are most likely due to the effects of CY rather than the treatment procedure.

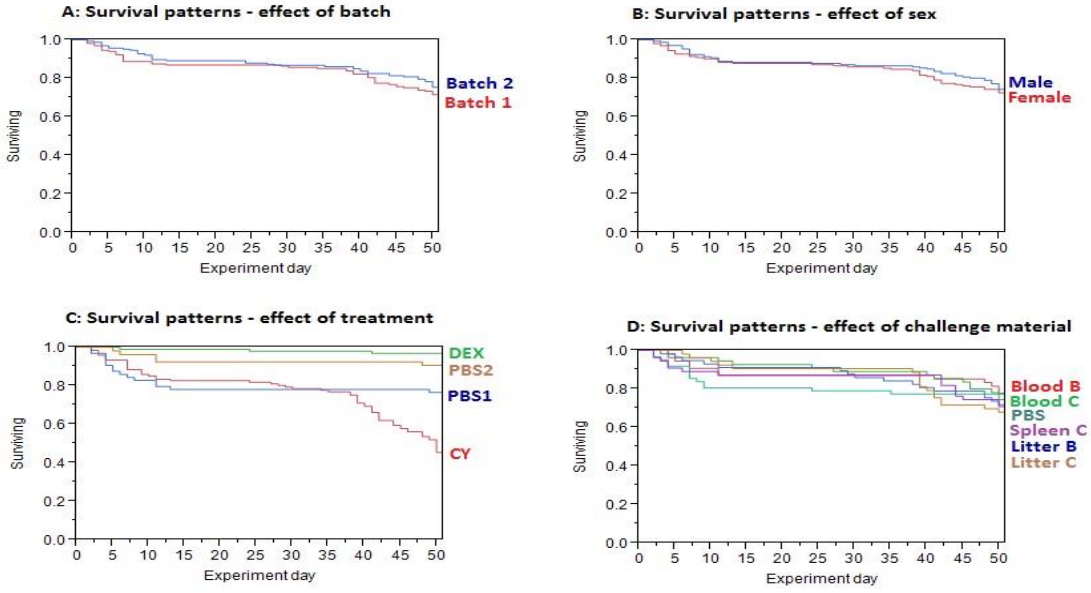

Figure 8.1 Survivability did not vary significantly due to chicken batch (A) ($P = 0.39$), sex (B) ($P = 0.63$) or challenge material (D) ($P = 0.89$). It did vary significantly with immunosuppressive treatment (C) ($P < 0.001$). CY and PBS1 treatments were administered on days of age 1-4 inclusive, DEX and PBS2 treatments on days of age 18-21 inclusive and challenge was on experiment day 21.

As is evident in Figure 8.1.C a large proportion of the CY group mortalities occurred following challenge and the majority after 35 days of age. Key features associated with these mortalities were lethargy and depression followed quickly by death (within 24 hours). On PM examination the majority of chickens demonstrated pale musculature, jaundice associated with the liver and coelomic cavity fat and haemorrhage associated with the liver, kidney and musculature, with examples provided in Figure 8.2.

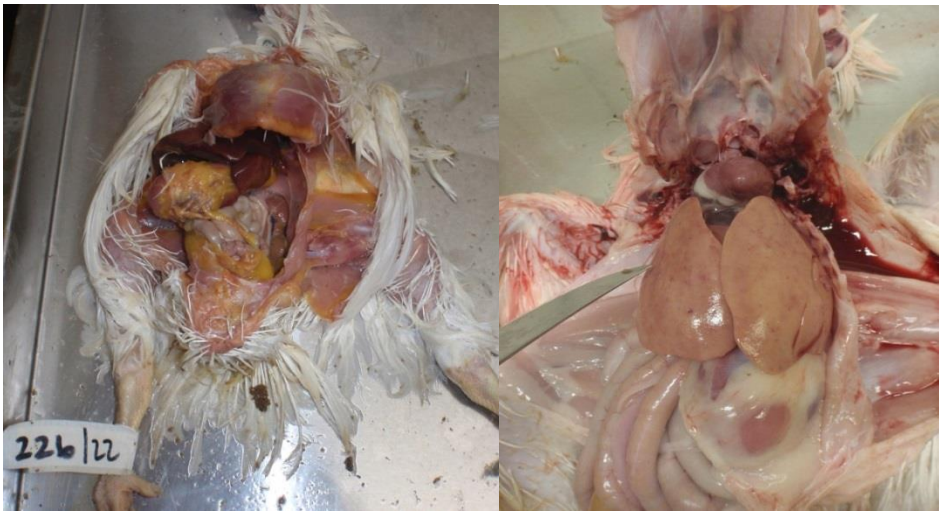

Figure 8.2 Showing pronounced jaundice of coelomic fat in a broiler on Expt d50 (left panel), pale and jaundiced appearance to the liver in a broiler on Expt d51 (right panel).

A survival analysis was conducted considering only CY treatment chickens and excluding all mortalities occurring prior to challenge, from which it was determined that the effect of challenge material on survivability was significant ($P < 0.01$) and the survivability of both litter challenge material groups was the lowest, as is shown in Figure 8.3.

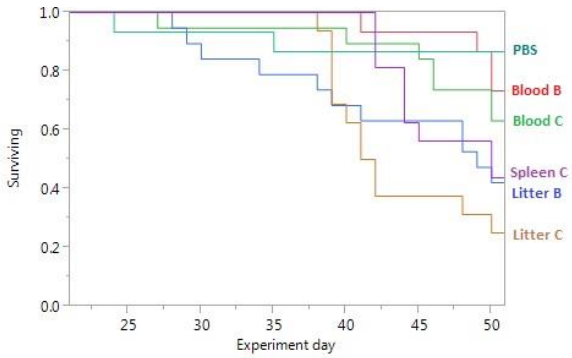

Figure 8.3 Survival curve between experiment days 21 and 51 for cyclophosphamide treated chickens. This time period excludes all mortalities prior to challenge. Challenge material had a significant effect on survivability ($P < 0.01$).

8.3.2 Bodyweights

The summary of the main factors affecting bodyweight are that immunosuppressive treatment had a significant effect at both 36 ($P < 0.001$) and 51 ($P < 0.001$) days of age, challenge had a significant effect at 51 days of age ($P < 0.001$), batch 2 chickens were significantly heavier ($P = 0.001$) than batch 1 chickens at 51 days of age and there were significant interactions between the effects of chicken batch and challenge material at 36 days of age ($P = 0.007$) and between chicken batch and sex at 51 days of age ($P = 0.003$). At placement the mean bodyweights for batch 1 and batch 2 chickens were 42.8 g and 42.5 g respectively.

8.3.2.1 Bodyweight at 36 days of age

The mean bodyweights were 1339.7±37.0 g for batch 1 chickens (males 1357.3±58.1 g, females 1322.2±52.1 g) and 1372.0±37.6 g for batch 2 chickens (males 1359.7±54.5 g, females 1384.4±50.3 g) and the difference between each batch was non-significant ($P = 0.58$).

The mean bodyweights for CY, PBS1, DEX and PBS2 treatment groups were 1146±70 g, 1370±54 g, 1397±38 g, and 1510±39 g respectively and the effect of immunosuppressive treatment was significant ($P < 0.001$). These results are shown in Figure 8.4 (left panel) from which it is evident that the mean bodyweight for CY treatment chickens was significantly lower than for DEX and PBS2 treatment groups but not PBS1 groups. Thus the early immunosuppressive treatment handling procedure for CY and PBS1 treatment groups likely influenced bodyweight at this point which may be in addition to any pharmacological effects of CY. There was a significant interaction between the effects of chicken batch and challenge material on bodyweight at 36 days of age ($P = 0.007$) and this is shown in Figure 8.4 (right panel) relevant to the litter C challenge. The effects of sex ($P = 0.93$), challenge material ($P = 0.94$) and isolator ($P = 0.30$) on bodyweight at 36 days of age were all non-significant ($P = 0.30$).

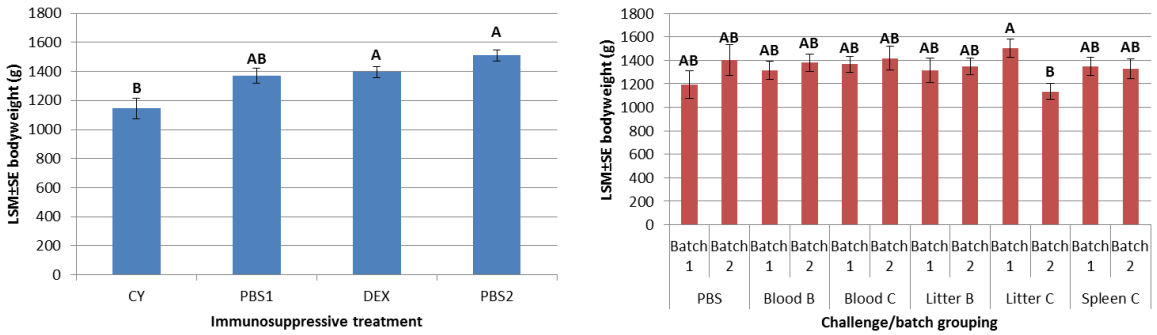

Figure 8.4 Mean bodyweight (LSM ± SE) on Expt d36 (n = 144) by immunosuppressive treatment (left panel) and challenge material/batch combination (right panel). Columns not sharing a common letter differ significantly.

8.3.2.2 Bodyweight at 51 days of age

The mean bodyweights for batch 1 and batch 2 chickens were 2185±40 g (males 2142±57 g, females 2228±58 g) and 2367±40 g (males 2490±54 g, females 2244±59 g) respectively and the difference between each batch was significant (P = 0.001).

The effects of immunosuppressive treatment (P < 0.001) and challenge material (P < 0.001) on bodyweight were both significant, with the CY treated chickens being significantly lighter than any other treatment group (Figure 8.5 left panel) and the Litter C challenged chickens being the lightest of all challenge groups (Figure 8.5 right panel).

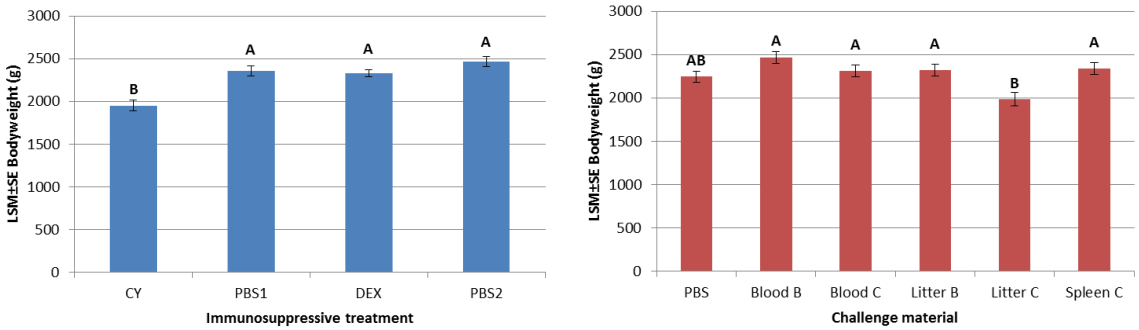

Figure 8.5 Mean bodyweight (LSM ± SE) on Expt d51 (n = 247) by immunosuppressive treatment (left panel) and challenge material (right panel). Columns not sharing a common letter differ significantly.

There was significant interaction between the effects of sex and batch on bodyweight (P = 0.003) (Figure 8.6 left panel) as males from batch 2 were significantly heavier than any other grouping. There was no significant overall interaction between the effects of batch and challenge material (P = 0.22); however, means separation by Tukey's HSD revealed that the bodyweight of batch 1 chickens challenged with Litter C was significantly lower than most other groupings (Figure 8.6 right panel). The effects of sex (P = 0.19) and isolator (P = 0.12) on bodyweight were both non-significant.

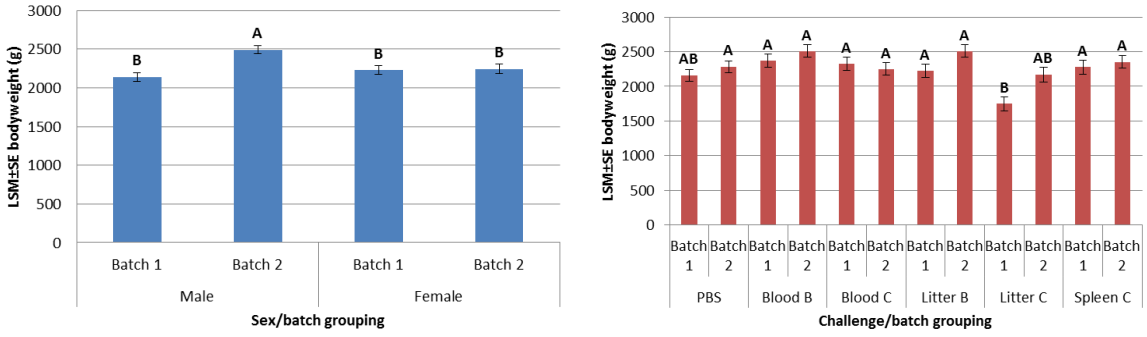

Figure 8.6 Mean bodyweight (LSM ± SE) by sex and chicken batch combination (left panel) and challenge material and batch combination (right panel) on Expt d51 (n = 247). Columns not sharing a common letter differ significantly.

8.3.3 Bursa of Fabricius

The summary of the main factors affecting relative bursa of Fabricius weight are that immunosuppressive therapy had a significant effect at both 36 ($P < 0.001$) and 51 ($P = 0.004$) days of age, challenge had a significant effect ($P = 0.03$) at 51 days of age, males recorded significantly higher ($P = 0.002$) mean relative bursal weights than females at 51 days of and there was a significant interaction between the effects of chicken batch and challenge material at 51 days of age ($P = 0.024$).

8.3.3.1 Relative bursa of Fabricius weight at 36 days of age

The mean relative bursal weights for batch 1 and batch 2 chickens were 0.1331±0.0070 % and 0.1291±0.0071 % respectively and the difference between each was non-significant ($P = 0.68$).

Immunosuppressive treatment had a significant effect on relative bursal weight ($P < 0.001$) with mean relative bursal weights for CY, PBS1, DEX, and PBS2 treatment groups measuring 0.048±0.013 %, 0.173±0.010 %, 0.156±0.007 %, and 0.147±0.008 % respectively. The relative bursal weight of CY treatment chickens was significantly lower than other immunosuppressive treatment groups (Figure 8.7 left panel). The effects of sex ($P = 0.08$), challenge material ($P = 0.20$), isolator ($P = 0.70$) and PM operator ($P = 0.25$) on relative bursal weight were all non-significant.

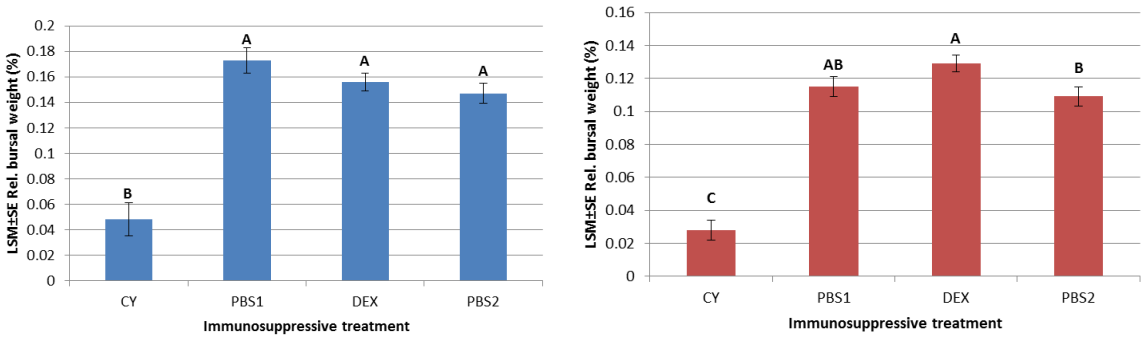

Figure 8.7 Mean relative bursal weight (LSM ± SE) by immunosuppressive treatment on Expt d36 (left panel) (n = 144) and Expt d51 (right panel) (n = 247). Columns not sharing a common letter differ significantly.

8.3.3.1 Relative bursa of Fabricius weight at 51 days of age

The mean relative bursal weights for batch 1 and batch 2 chickens were 0.1030±0.0040 % and 0.0870±0.0040 % respectively and the difference between each was significant ($P = 0.004$).

The effect of immunosuppressive treatment on relative bursal weight was significant ($P < 0.0001$) and consistent with findings on Expt d36 in relation to the effect of CY treatment as shown in Figure 8.7 (right panel). An additional finding on Expt d51 was that the relative bursal weight of DEX treatment chickens was significantly higher than the PBS2 control (Figure 8.7). Figure 8.8

shows a sample of 3 bursae of Fabricius which were typical of what was observed from CY treatment chickens compared to bursae of Fabricius observed from PBS1 treated chickens.

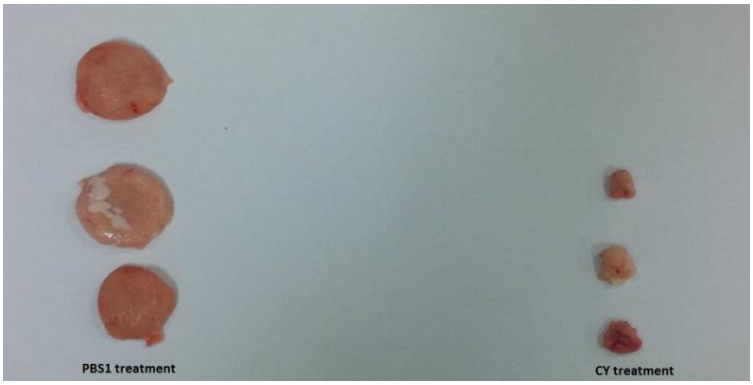

Figure 8.8 Examples of bursae of Fabricius on Expt d51 from PBS1 (left) and CY treatment chickens (right).

The effect of challenge material on relative bursal weight was significant (P = 0.031) and as is evident in Figure 8.9 (left panel) the relative bursal weight for Litter C challenged chickens was significantly greater than that for Blood C challenged chickens. The mean relative bursal weight of males 0.1047±0.0041 % was significantly greater than that of females 0.0851±0.0043 % (P = 0.002). The effect of the interaction between challenge material and batch on relative bursal weight on Expt d51 was significant (P = 0.024) and this is displayed in Figure 8.9 (right panel) from which it is evident relative bursal weights of chickens challenged with Blood B varied significantly between chicken batches. The effects of isolator (P = 0.99) and PM operator (P = 0.19) on relative bursal weight were both non-significant.

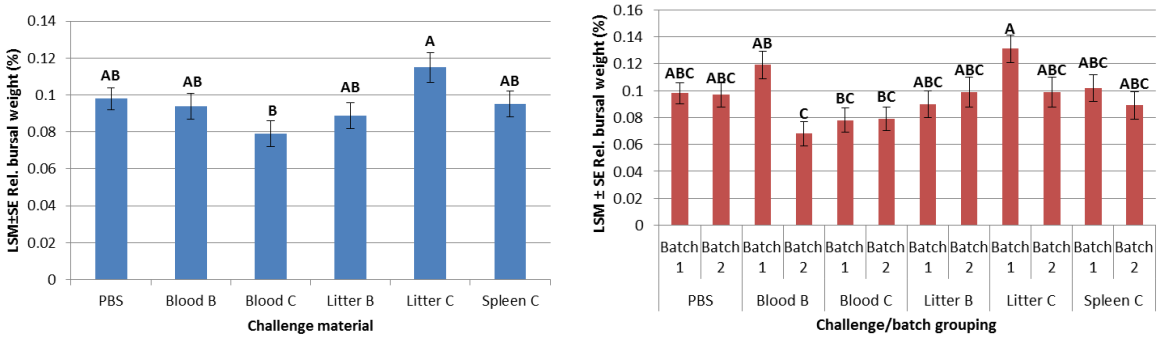

Figure 8.9 Relative bursal weight (LSM ± SE) by challenge material (left panel) and challenge material/batch combination (right panel) on Expt d51 (n = 247). Columns not sharing a common letter differ significantly.

8.3.4 Spleen

The summary of the main factors affecting relative spleen weight are that challenge material had a significant effect at both 36 ($P < 0.001$) and 51 ($P < 0.001$) days of age, males recorded significantly higher ($P = 0.02$) mean spleen weights than females at 51 days of age and the interactions between the effects of chicken batch and challenge material at 36 days of age ($P = 0.03$) and immunosuppressive treatment and challenge material at 51 days of age ($P = 0.04$) on relative spleen weight were both significant.

8.3.4.1 Relative spleen weight at 36 days of age

The mean relative spleen weight for batch 1 and batch 2 chickens were 0.0819±0.0045 % and 0.0861±0.0046 % respectively and the difference between each was non-significant ($P = 0.49$). Challenge material had a significant effect on relative spleen weight on Expt d36 ($P < 0.001$) with the variation between groups shown in Figure 8.10 (left panel). The effect of the interaction between challenge material and batch on relative spleen weight was also significant ($P = 0.03$) and the variation between groups is shown in Figure 8.10 (right panel). The effects of sex ($P = 0.65$), immunosuppressive treatment ($P = 0.78$), isolator ($P = 0.60$) and PM operator ($P = 0.57$) on relative spleen weight were all non-significant.

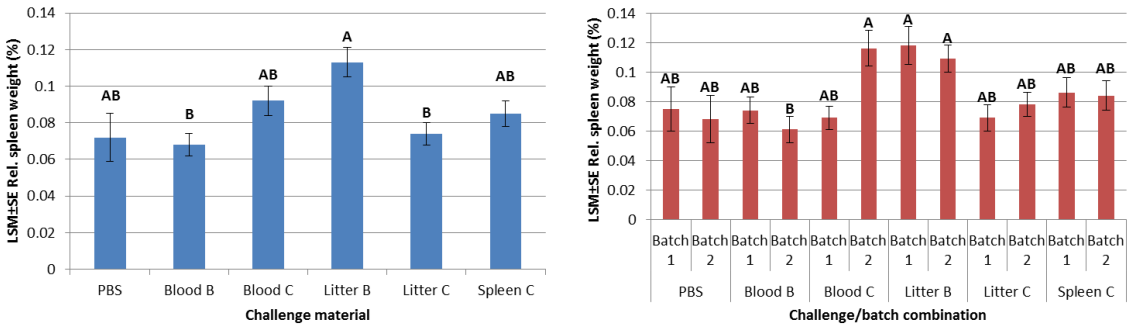

Figure 8.10 Relative spleen weight (LSM ± SE) by challenge material (left panel), and challenge material/batch combination (right panel) on Expt d36 (n = 144). Columns not sharing a common letter differ significantly.

8.3.4.1 Relative spleen weights at 51 days of age

On Expt d51 the mean relative spleen weights for batch 1 and batch 2 chickens was 0.0926±0.0028 % and 0.0968±0.0024 % respectively and the difference between each was non-significant ($P = 0.25$). Challenge material had a significant effect ($P < 0.001$) on relative spleen weight on Expt d51 as shown in Figure 8.11 with the relative spleen weights of Blood C challenged chickens being significantly heavier than that for other challenge material groupings.

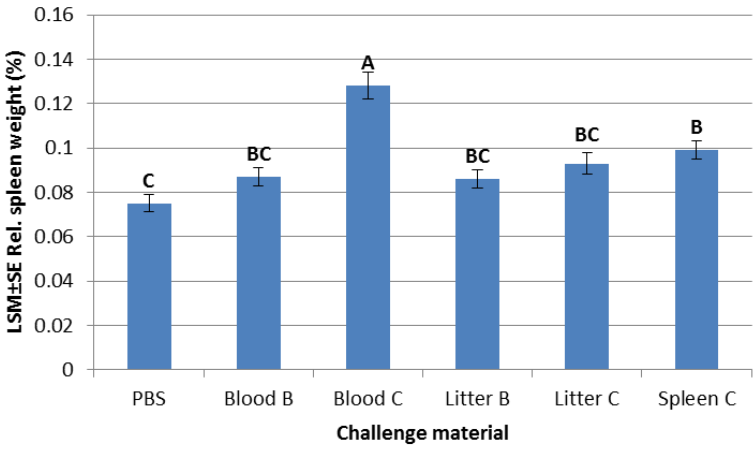

Figure 8.11 Relative spleen weight (LSM ± SE) by challenge material on Expt d51 (n = 247). Columns not sharing a common letter differ significantly.

Sex had a significant effect (P = 0.02) on mean relative spleen weight (males 0.0991±0.0026 %, females, 0.0902±0.0027 %). The effect of the interaction between challenge material and immunosuppressive treatment on relative spleen weight was significant (P = 0.04); however, there was not any systematic effect of one treatment over another. The effects of immunosuppressive treatment (P = 0.61), isolator (P = 0.18) and PM operator (P = 0.33) on relative spleen weight were all non-significant.

8.3.5 Histopathology

A selection of 10 fixed liver samples from mortalities demonstrating jaundice of coelomic fat and/or liver between experiment days 41-48 were sent to the SVDL for histological evaluation and resultant findings are outlined in Table 8.3. A total of six of these samples demonstrated eosinophilic intra-nuclear inclusion bodies within hepatocytes, consistent with inclusion body hepatitis from fowl adenovirus infection.

Table 8.3 Liver histopathological findings from a selection of mortalities from experimental days 41-48 (interpretations provided by Dr. Rod Reece).

Bird #	Day	Treatment	Challenge material	Histopathological findings
202	41	CY	Litter B	Diffuse mild fatty changes, small portal foci of mixed inflammatory cells.
203	41	CY	Litter C	Many hepatocytes have small fatty vacuoles, a small number of scattered but definitive eosinophilic intra-nuclear inclusion bodies (E-INICB).
206	41	CY	Litter C	A few foci of portal mixed inflammatory cells, a few E-INICB.
210	42	CY	Litter C	Multiple foci of degenerate hepatocytes with moderate fatty change and numerous well-formed E-INICB.
212	44	CY	Spleen C	Mild to moderate fatty change, focal hepatic necrosis with some small foci of inflammatory cells, numerous E-INICB.
213	44	CY	Spleen C	Mild-moderate mixed inflammatory cell infiltrate.
214	44	CY	Spleen C	Massive foci of confluent necrosis, fatty change in hepatocytes, scattered E-INICB.
216	45	CY	Blood C	Moderate diffuse fatty change, scattered individual hepatocyte necrosis.
218	46	CY	Blood C	Moderate fatty change.
219	48	CY	Litter C	Confluent foci of degeneration, moderate fatty change, numerous E-INICB.

CY = cyclophosphamide.

Additionally, fixed sections of brain and spinal cord segments (cervical, thoracic and lumbar) from clinically normal chickens (PBS or blood C challenged) sampled on Expt d51 were submitted to the SVDL for histological evaluation. The histopathological findings which are summarised in Table 8.4 demonstrate that Wallerian degeneration, characterised by chains of digestion chambers containing axonal debris, could be observed throughout the spinal cord in both PBS and blood challenged chickens. In some of the sections observed, the Wallerian degeneration increased in severity further caudally in that chicken.

Table 8.4 Central nervous system histopathological findings from a selection of chickens on Expt d51 (interpretations provided by Dr. Rod Reece).

Bird #	Treatment	Challenge material	Histopathological findings
460	DEX	Blood B	**Brain:** no significant findings. **Cervical cord:** scattered vacuoles, some myelinomacrophages, several segmentally swollen axons. **Thoracic cord:** similar in appearance to cervical cord. **Lumbar Cord:** vacuolation present, chains of digestion chambers containing cellular debris present (Wallerian degeneration).
461	PBS2	Blood B	**Brain:** several vacuoles in the optic tectum. **Cervical cord:** several small foci of Wallerian degeneration, vacuolation evident, some macrophages present. **Thoracic cord:** moderate amount of Wallerian degeneration present. **Lumbar cord:** moderate amount of Wallerian degeneration present.
462	CY	Blood B	**Brain:** no significant findings. **Cervical cord:** extensive Wallerian degeneration present. **Thoracic cord:** extensive Wallerian degeneration present with readily recognisable degenerate macrophages. **Lumbar cord:** marked and extensive Wallerian degeneration present, note Wallerian degeneration gets worse in this bird further caudally.
469	PBS2	PBS	**Brain:** no significant findings. **Cervical cord:** moderate and extensive Wallerian degeneration with multiple severely swollen axons. **Thoracic cord:** similar findings to cervical cord. **Lumbar cord:** similar findings to cervical and thoracic cords, gets worse further caudally.
470	CY	PBS	**Brain:** no significant findings. **Cervical cord:** a few distinct vacuoles with degenerate macrophages. **Thoracic cord:** numerous vacuoles and foci of Wallerian degeneration. **Lumbar cord:** extensive vacuolation.
471	DEX	PBS	**Brain:** no significant findings. **Cervical cord:** extensive vacuolation and Wallerian degeneration with degenerate macrophages. **Thoracic cord:** similar findings to cervical cord. **Lumbar cord:** similar findings to cervical and thoracic cords but more marked, Wallerian degeneration gets worse further caudally in this bird.

CY = cyclophosphamide, DEX = dexamethasone, PBS = phosphate buffered saline.

8.3.6 Serology

Presence of maternal antibody directed against a range of viral pathogens for each batch were measured from serum extracted from chickens at the time of placement of each batch (Table 8.5). Seropositivity was detected in the majority of samples tested in both batches for AEV, IBDV, FAV8 and CAV. A significant difference was detected between batches for mean optical density (OD) readings for FAV8, with positive ODs for batch 1 being higher than for batch 2. The differences in positive titres between batches for AEV, IBDV, Reo and CAV were not significant.

Table 8.5 Maternal antibody status for a number of key viral diseases at placement (Expt days 0 and 1) by batch and virus.

Batch		AEV	IBDV	FAV	Reo	CAV
1	Percentage positive (from 10 samples)	100 %	100 %	80 %	30 %	80 %
	Mean positive titre (±SE)	3266±684	10136±1009	2.60±0.20*	1454±360	7500±475
	Mean negative titre (±SE)	n/a	n/a	0.77*	242±24	990±0
2	Percentage positive (from 10 samples)	80 %	100 %	100 %	20 %	100 %
	Mean positive titre (±SE)	3538±393	9355±858	1.88±0.16*	606±904	6514±903
	Mean negative titre (±SE)	278±13	n/a	n/a	162±19	n/a
Effect of batch on proportion positive (P =)		0.75	0.56	**0.01**	0.17	0.38

*Results reported as OD reading as titre not available. AEV = avian encephalomyelitis virus, IBDV = infectious bursal disease virus, FAV8 = fowl adenovirus serotype 8, Reo = reovirus, CAV = chicken anaemia virus.

On Expt d51 none of the samples that were tested were seropositive to IBDV (Table 8.6). A small proportion of Spleen C challenged chickens were seropositive to AEV (Table 8.6). A large proportion of Litter B challenged chickens in all immunosuppressive treatment groups except CY and a small proportion of Litter C challenged chickens from the PBS1 treatment group were FAV8 seropositive (Table 8.6). The effect of immunosuppressive treatment on seropositivity to FAV8 was significant ($P = 0.01$) which was due to failure to seroconvert to FAV8 in any CY treated chicken (Table 8.6). Additionally the effect of challenge material on seropositivity to FAV8 was significant ($P < 0.01$) due to the lack of seroconversion in Blood B, Blood C, Spleen C and PBS challenged chickens and Litter C inducing seroconversion in a small proportion of chickens compared to very high proportions of positive chickens from Litter B groups (Table 8.6).

A proportion of chickens challenged with Litter B or Litter C were positive to Reo from all immunosuppressive treatment groups except CY (Table 8.6). The effect of batch on seropositivity to Reo was significant, with a higher proportion of batch 1 chickens being seropositive (Table 8.6). Varying proportion of chickens were seropositive to CAV through all immunosuppressive treatment and challenge material combinations, except CY treated chickens challenged with Litter B (Table 8.6). These results suggest that vertical transmission of CAV occurred to a level given that PBS challenged chickens were also seropositive.

Table 8.6 Antibody status (percentage positive) on Expt d51 by immunosuppressive treatment, challenge material and virus.

Treatment	Challenge material	No. of samples	AEV	IBDV	FAV8	Reo	CAV
CY	PBS	4	0 %	0 %	0 %	0 %	50 %
	Blood B	3	0 %	0 %	0 %	0 %	33 %
	Litter B	4	0 %	0 %	0 %	0 %	0 %
	Blood C	4	0 %	0 %	0 %	0 %	50 %
	Spleen C	3	0 %	0 %	0 %	0 %	33 %
	Litter C	3	0 %	0 %	0 %	0 %	33 %
PBS1	PBS	3	0 %	0 %	0 %	0 %	33 %
	Blood B	4	0 %	0 %	0 %	0 %	50 %
	Litter B	5	0 %	0 %	80 %	20 %	60 %
	Blood C	4	0 %	0 %	0 %	0 %	100 %
	Spleen C	4	25 %	0 %	0 %	0 %	25 %
	Litter C	4	0 %	0 %	25 %	50 %	50 %
DEX	PBS	3	0 %	0 %	0 %	0 %	67 %
	Blood B	5	0 %	0 %	0 %	0 %	40 %
	Litter B	4	0 %	0 %	100 %	50 %	50 %
	Blood C	4	0 %	0 %	0 %	0 %	50 %
	Spleen C	5	20 %	0 %	0 %	0 %	60 %
	Litter C	5	0 %	0 %	0 %	20 %	80 %
PBS2	PBS	4	0 %	0 %	0 %	0 %	25 %
	Blood B	4	0 %	0 %	100 %	67 %	50 %
	Litter B	3	0 %	0 %	0 %	0 %	67 %
	Blood C	4	0 %	0 %	0 %	0 %	75 %
	Spleen C	4	0 %	0 %	0 %	0 %	75 %
	Litter C	4	0 %	0 %	0 %	0 %	100 %
Mean positive titre (±SE)			636±91	n/a	1.68±0.14*	811±82	2972±380
Mean negative titre (±SE)			68±8	54±4	0.21±0.02*	86±11	999±0
Effect of treatment (P =)			1.00	1.00	**0.01**	0.21	0.18
Effect of challenge material (P =)			0.88	1.00	**<0.01**	0.15	0.41
Effect of sex (P =)			0.12	1.00	1.00	1.00	0.13
Effect of batch (P =)			1.00	1.00	1.00	**0.02**	1.00
Effect of isolator (P =)			1.00	1.00	1.00	1.00	0.11

*Results reported as OD reading as titre not available. AEV = avian encephalomyelitis virus, CAV = chicken anaemia virus, CY = cyclophosphamide, DEX = dexamethasone, FAV8 = fowl adenovirus serotype 8, IBDV = infectious bursal disease virus, PBS = phosphate buffered saline, Reo = reovirus.

8.3.7 Molecular diagnostics

Molecular diagnostics for FAV8 and CAV were performed to investigate the significant level of mortality observed in CY treated chickens from day 35, most of which demonstrated signs of jaundice associated with the liver and coelomic fat and paleness of the liver and musculature.

8.3.7.1 PCR for the detection of FAV8

A standard PCR for the detection of FAV8 was performed on DNA extracted from livers from a selection of mortalities (Table 8.7). FAV8 DNA was detected in three chickens (Table 8.7). Figure 8.12 is a photograph of an agarose gel from this analysis.

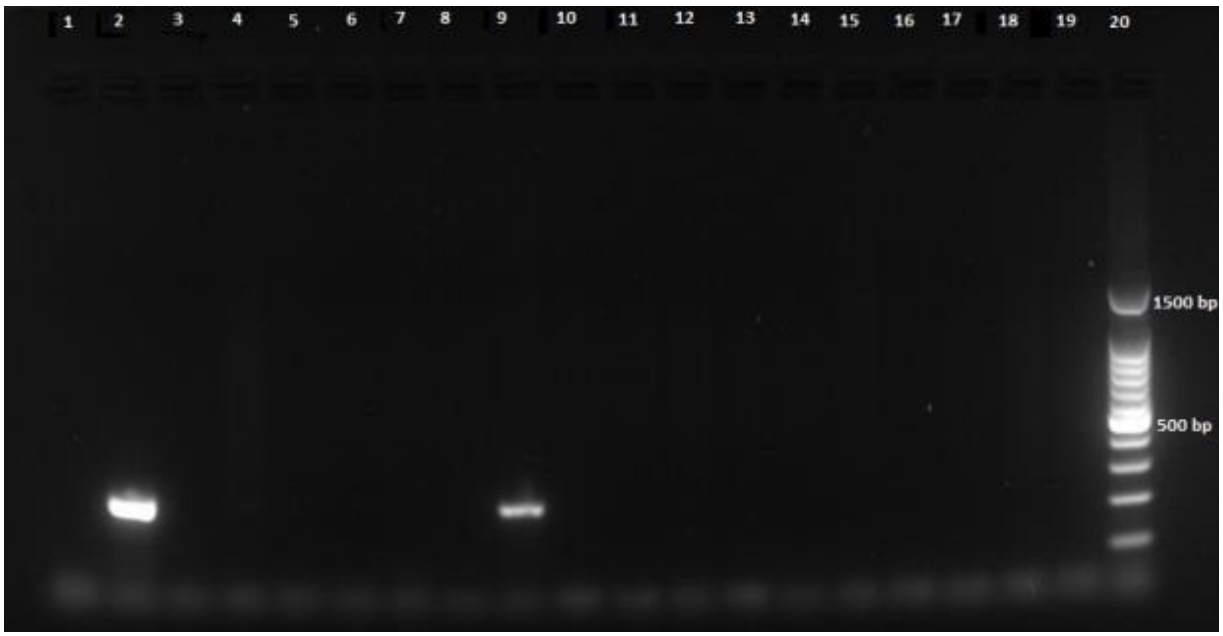

Figure 8.12 Agarose gel analysis of amplified DNA from FAV8 PCR. 1: No template control; 2: Positive control showing an intense band at 229 bp; 3-4: Birds 193-194; 5-8: Birds 196-199; 9-10: Birds 202-203; 11-19: Birds 206 - 214; 20: 100 bp DNA ladder.

8.3.7.2 PCR for the detection of CAV

A standard PCR for the detection of CAV was performed on DNA extracted from livers from a selection of mortalities (Table 8.7). CAV DNA could be detected in 12 of the 23 samples tested (Table 8.7). Figure 8.13 is a photograph of an agarose gel from this analysis.

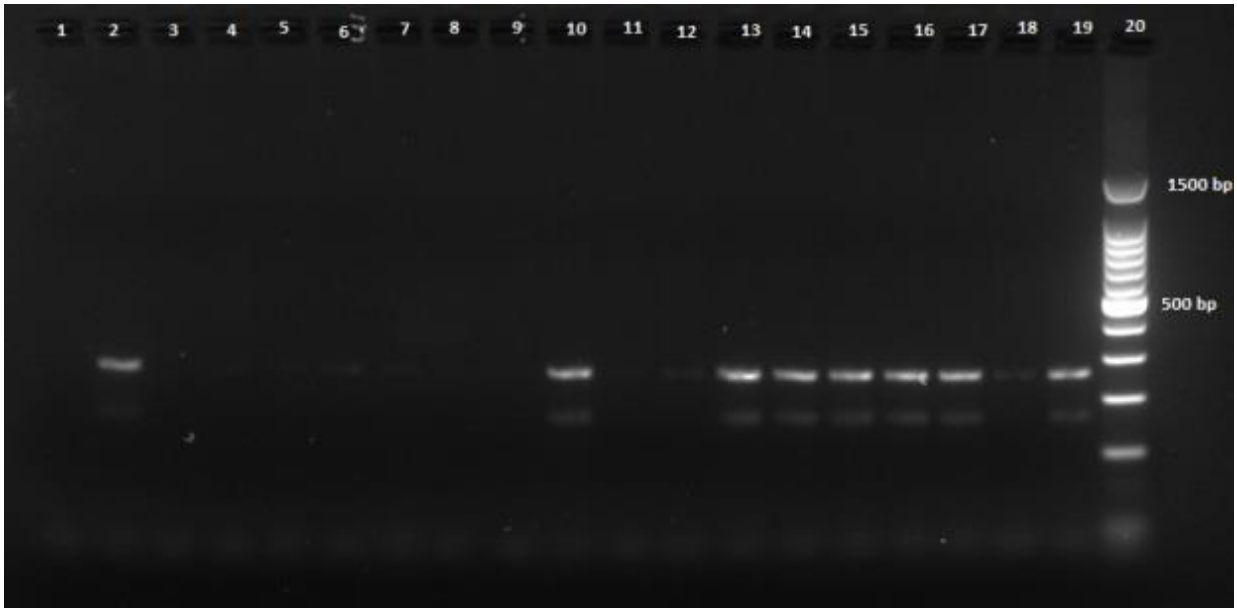

Figure 8.13 Agarose gel analysis of amplified DNA from CAV PCR. 1: No template control; 2: Positive control showing an intense band at 280 bp; 3-4: Birds 193-194; 5-8: Birds 196-199; 9-10: Birds 202-203; 11-19: Birds 206 - 214; 20: 100 bp DNA ladder.

A qPCR was performed using DNA extracted from livers from a range of chickens from the experiment (Table 8.7). A total of nine of the 26 Expt d51 samples were CAV positive and the same 12 of 23 samples from mortalities were CAV positive on qPCR (Table 8.7). CAV DNA was detected in the Spleen C challenge material, but not in either of the Blood B or Blood C challenge material using qPCR.

From analysis of CAV load (log_{10}-transformed VCN/ mg tissue) of all positive samples reported in Table 8.7, the effects of immunosuppressive treatment (P = 0.94), challenge (P = 0.23) and challenge nested within isolator (P = 0.54) on CAV load in the liver were non-significant. The mean log_{10}-transformed VCN/ mg tissue by immunosuppressive treatment is shown in Figure 8.14.

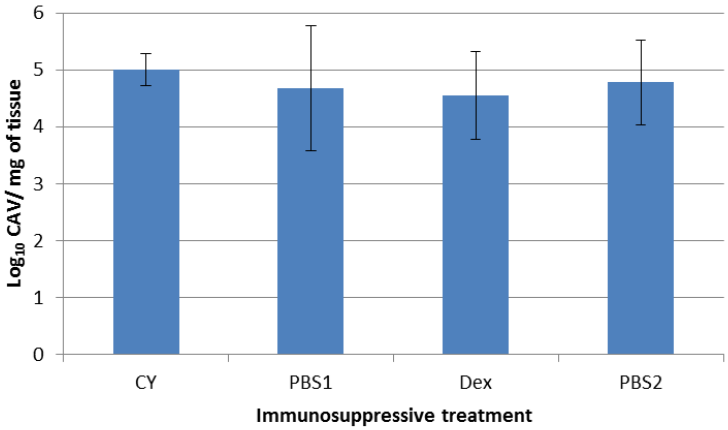

Figure 8.14 Log10 CAV/ mg liver tissue (harvested at time of mortality or at conclusion of the experiment) by immunosuppressive treatment group.

Table 8.7 Summary of results from molecular diagnostics by experimental bird number

Bird #	DNA source	Day	Batch	Treatment	Challenge	FAV8 PCR	CAV PCR	CAV qPCR
193	Liver	38	1	CY	Litter C	Negative	Negative	Negative
194	Liver	38	1	CY	Litter B	Negative	Negative	Negative
196	Liver	39	2	CY	Litter C	Negative	Negative	Negative
197	Liver	39	1	CY	Litter B	Negative	Negative	Negative
198	Liver	39	1	CY	Litter C	Negative	Negative	Negative
199	Liver	39	1	CY	Litter C	Negative	Negative	Negative
202	Liver	41	2	CY	Litter B	Positive	Negative	Negative
203	Liver	41	2	CY	Litter C	Negative	Positive	Positive
206	Liver	41	1	CY	Litter C	Negative	Negative	Negative
207	Liver	42	1	CY	Litter C	Negative	Negative	Negative
208	Liver	42	1	CY	Spleen C	Negative	Positive	Positive
209	Liver	42	1	CY	Spleen C	Negative	Positive	Positive
210	Liver	42	1	CY	Litter C	Negative	Positive	Positive
211	Liver	42	1	CY	Spleen C	Negative	Positive	Positive
212	Liver	44	2	CY	Spleen C	Negative	Positive	Positive
213	Liver	44	2	CY	Spleen C	Negative	Negative	Negative
214	Liver	44	1	CY	Spleen C	Negative	Positive	Positive
216	Liver	45	1	CY	Blood C	Negative	Positive	Positive
217	Liver	46	1	CY	Blood C	Negative	Positive	Positive
218	Liver	46	2	CY	Blood C	Negative	Positive	Positive
219	Liver	48	2	CY	Litter C	Negative	Positive	Positive
220	Liver	48	1	CY	Litter B	Negative	Positive	Positive
222	Liver	48	2	CY	Litter B	Positive	Positive	Positive
234	Liver	51	2	CY	Litter B	Positive	Negative	Negative
235	Liver	51	2	PBS1	Litter B	nt	nt	Negative
236	Liver	51	2	DEX	Litter B	nt	nt	Negative
276	Liver	51	2	PBS2	Spleen C	nt	nt	Negative
277	Liver	51	2	CY	Spleen C	nt	nt	Positive
278	Liver	51	2	DEX	Spleen C	nt	nt	Negative
308	Liver	51	1	CY	Blood B	nt	nt	Negative
309	Liver	51	1	DEX	Blood B	nt	nt	Negative
310	Liver	51	1	PBS1	Blood B	nt	nt	Negative
389	Liver	51	2	DEX	Blood C	nt	nt	Negative
390	Liver	51	2	CY	Blood C	nt	nt	Positive
391	Liver	51	2	PBS2	Blood C	nt	nt	Positive
419	Liver	51	2	PBS1	PBS	nt	nt	Negative
420	Liver	51	2	CY	PBS	nt	nt	Negative
421	Liver	51	2	DEX	PBS	nt	nt	Negative
440	Liver	51	1	PBS1	Litter C	nt	nt	Positive
441	Liver	51	1	DEX	Litter C	nt	nt	Positive
442	Liver	51	1	CY	Litter C	nt	nt	Positive
449	Liver	51	1	PBS2	Spleen C	nt	nt	Positive
450	Liver	51	1	CY	Spleen C	nt	nt	Positive
451	Liver	51	1	DEX	Spleen C	nt	nt	Positive
469	Liver	51	1	PBS2	PBS	nt	nt	Negative
470	Liver	51	1	CY	PBS	nt	nt	Negative
471	Liver	51	1	DEX	PBS	nt	nt	Negative

CAV = chicken anaemia virus, CY = cyclophosphamide, DEX = dexamethasone, FAV8 = fowl adenovirus serotype 8, PCR = polymerase chain reaction, PBS = phosphate buffered saline, nt = not tested.

8.3.9 Further effects of immunosuppressive treatments

8.3.9.1 Effect of CY on feather development

CY treated chickens developed 'feather breaks' which were first noticed on day 12 of the experiment. These breaks were most obvious in the primary and secondary wing and tail feathers and were not observed in chickens from any other immunosuppressive treatment group. Figure 8.15 highlights this observation (left panel) compared to a normal chicken (right panel).

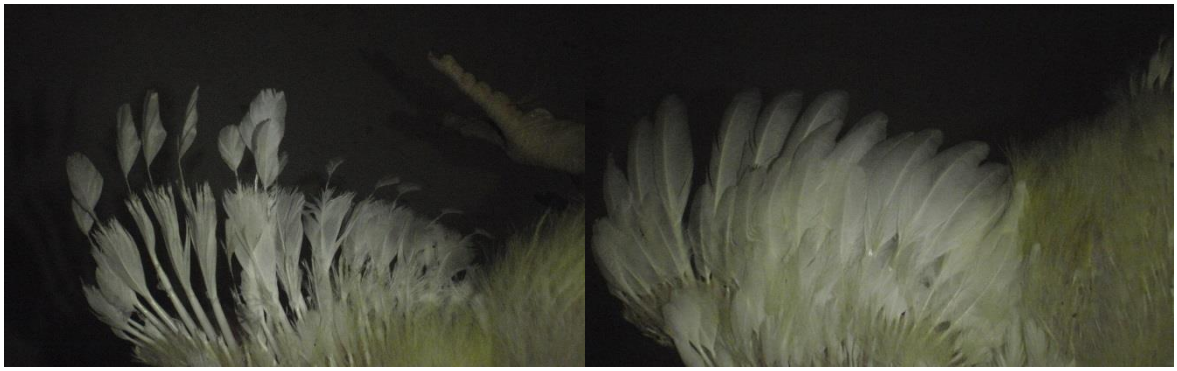

Figure 8.15 Feather breaks observed in wing feathers of a cyclophosphamide treated chicken (left panel) compared to the normal wing feathering of a PBS1 treated chicken on day 18 of the experiment.

8.3.9.2 Effects of immunosuppression on circulating white blood cell levels

There was no significant difference in total circulating white blood cells and total and relative levels of circulating neutrophils, lymphocytes, monocytes, eosinophils and basophils between immunosuppressive treatment groups on Expt d36 (Table 8.8).

Table 8.8 LSM total (x106/mL) and relative (%) levels of circulating white blood cells by immunosuppressive treatment group

Treatment	WBC ($x10^6$/mL)	Neut. ($x10^6$/mL)	Neut. (%)	Lymph. ($x10^6$/mL)	Lymph. (%)	Mon. ($x10^6$/mL)	Mon. (%)	Eos. ($x10^6$/mL)	Eos. (%)	Baso. ($x10^6$/mL)	Baso. (%)
CY	50.8	8.96	17.9	31.4	62.5	7.8	14.6	0.01	0.03	2.7	5.1
PBS1	52.0	7.70	16.1	22.4	44.6	13.0	23.4	0.01	0.01	8.8	16.0
DEX	57.4	8.15	16.5	25.8	52.2	14.5	19.7	0.01	0.01	9.1	11.6
PBS2	39.2	9.66	24.8	24.7	62.6	3.9	9.8	0.01	0.01	0.9	2.4
Effect of treatment (P =)	0.13	0.66	0.12	0.11	0.14	0.18	0.18	0.86	0.12	0.15	0.18

Baso = basophils, CY = cyclophosphamide, DEX = dexamethasone, Eos = eosinophils, Lymph = lymphocytes, Mon = monocytes, Neut = neutrophils, PBS = phosphate buffered saline, WBC = white blood cells.

8.3.9.3 Effect of dexamethasone on serum glucose

Figure 8.16 shows the mean serum glucose concentrations for DEX (26.6±1.2 mmol/L) and PBS2 (14.7±2.0 mmol/L) treated chickens on Expt d21, on the last day of DEX treatment. Treatment with dexamethasone resulted in significantly higher serum glucose concentrations ($P < 0.001$) compared to similar treatment with PBS. The mean serum glucose concentration of PBS2 treated chickens fell within the reference range reported by Goodwin *et al.* (1994) (13.7±3.2 mmol/L).

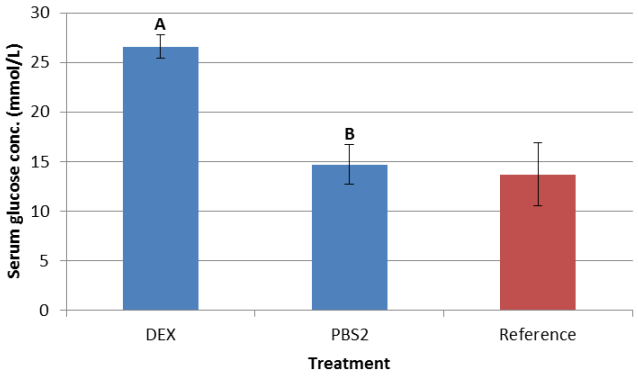

Figure 8.16 Mean serum glucose concentrations (mmol/L) of DEX and PBS2 treated chickens measured on Expt d21, compared to the reference range reported by Goodwin et al. (1994). Columns not sharing a common letter differ significantly.

8.4 Discussion

The design of this experiment (14-C-REP5) aimed to account for the differences in parent flock and challenge material source in the previously reported experiments which may explain the inability to reliably reproduce the APS. The experiment also used two immunosuppressive techniques in an attempt to further enhance the chances of reproducing the APS. Despite these strategies the APS was not reproduced in this experiment.

In previous experiments only single batches of chickens have been used. It is possible that different parent flocks provide different levels of maternal immunity or host resistance associated with APS. Taking this into account this experiment used two batches of chickens, each from different parent flocks (batch 1 – parent flock D, batch 2 – parent flock B). The batch 2 chickens were from the same parent flock as chickens from 13-C-REP1 in which the APS was reproduced. Thus parent flock has likely not played a role in the APS not being able to be reliably reproduced experimentally.

In order to reduce the risk of not being able to reproduce the APS due to challenge material containing insufficient or none of the putative infective agent/s, Blood B challenge material was used (in addition to Litter B harvested from the same case) which was used to reproduce the APS in one chicken in 13-C-REP4 reported in Chapter 7. This did not lead to reproduction of the syndrome, raising the possibility of differences in host chickens being responsible for the failure to reliably reproduce the APS experimentally.

Field observations and the epidemiological study (Chapter 5) have identified rapid growth as a risk factor for APS with the disease most observed in fast growing chickens, particularly males. It is therefore instructive to compare the growth rates of chickens in the different experiments attempting to reproduce the syndrome. Figure 8.17 highlights that mean bodyweights measured in

this experiment were lower than those achieved in both experiments in which the APS was successfully reproduced (13-C-REP1 and 13-C-REP4) and the Ross 308 performance guidelines (Aviagen, 2012). Thus the lower growth rates achieved in this experiment pose a possible reason for the failure to reproduce the APS. This is consistent with epidemiological analyses reported in Chapter 5.

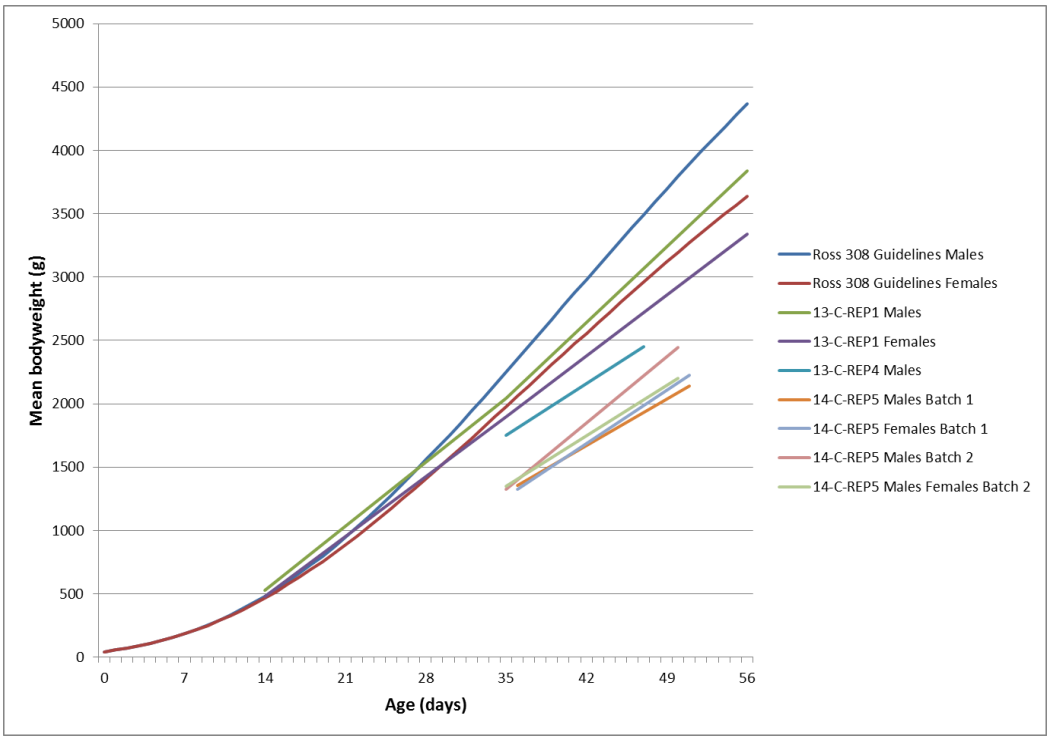

Figure 8.17 Mean bodyweights achieved by sex and batch as compared to the Ross 308 guidelines (Aviagen, 2012) and results from the experiment reported in Chapter 6 (13-C-REP1) and experiment 3 reported in Chapter 7 (13-C-REP3).

The isolator flooring design was different in this experiment compared to the perforated stainless steel flooring design used in the experiments in which the syndrome was successfully reproduced. The use of solid flooring trays with bedding material in them in this experiment was modified from that reported in Chapter 7 by the use of rice hulls instead of wood shavings and feeders being raised slightly to prevent litter spilling over into the feed trough. The reasons for using solid floors with rice hull bedding material were to reduce the incidence of lameness, thus improving chicken welfare, and to mimic the use of rice hulls as litter because all broiler growing farms in the region in which the APS occurs use rice hulls as shed litter. It is likely that the installation of the litter trays reduced the air quality within the isolators as the air in-flow ports were located above the floor and the out-flow ports below the floor, thus the solid flooring reduced air exchange and thus likely reduced air quality. It is conceivable that reduced growth rates result from a reduction in air quality.

From survival analyses, it is evident that survivability of chickens was reduced by the process of handling and administering IA injections in the first 4 days from placement. Additionally it was evident that survivability was reduced from the CY treatment and subsequent challenge with shed litter, spleen cells or whole blood, with the majority of resultant mortalities commencing from Expt d35.

The clinical syndrome described for the majority of these chickens prompted diagnostics to be performed to confirm the presence of CAV and FAV8 in a proportion of these chickens. FAV8 could not be detected by PCR in all of the liver samples tested in which histopathology consistent with inclusion body hepatitis was found. It is proposed that in this experiment the mortalities occurring from Expt d35 in immunosuppressed chickens were caused by at least in part, the actions of FAV8 and CAV, alone or in combination, due to the pattern of mortalities post-challenge, clinical signs, histopathological findings and presence of either agent as confirmed by PCR and qPCR.

The presence of CAV and FAV8 in challenge material is consistent with findings reported in Chapter 6 in which SPF chickens were seropositive to FAV8 from challenge with spleen and litter and to CAV from challenge with blood, spleen and litter. Litter was the only challenge material that induced seroconversion to FAV8 and predominantly from Litter B. However, these results are inconsistent with histopathology results whereby two Spleen C chickens displayed E-INICB consistent with FAV8 infection.

Treatment with CY inhibited seroconversion to FAV8 and Reo after challenge at 21 days of age which is consistent with CY induced suppression of humoral immunity. However, the same finding was not observed for CAV, as variable proportions of CY treated chickens seroconverted to CAV, which was indifferent to other immunosuppressive treatment groups. A potential reason for this is that CAV was likely transmitted vertically in a proportion of chickens, thus the humoral immune response to CAV had likely commenced just prior to or at the time of CY treatment. Research defining the interference caused by the suppression of humoral immunity closely following infectious challenge is unavailable to support this theory.

In addition to affecting survivability, CY treatment had a significant negative effect on bodyweight. In relation to bodyweight, batch 2 chickens performed significantly better than batch 1 chickens. One reason for this could be due to different parent flocks, if the maternal antibody coverage provided to batch 1 was insufficient for complete protection against agent/s present in the challenge material, subsequently affecting performance. Batch 1 chickens challenged with Litter C recorded significantly lower Expt d51 bodyweights compared to most groups.

Furthermore the Litter C challenged chickens recorded the highest relative bursal weights on Expt d51, mostly in batch 1 chickens, which is consistent with immune challenge from this material. Environmental factors including incubation and handling at the hatchery and during transport could also be implicated in variances in performance between batches.

CY treatment reduced relative bursal weights compared to PBS treated controls as measured on both Expt d36 and Expt d51. This finding is consistent with the prolonged suppressive effect that CY is known to have on the B-cell immune system of chickens (Lerman & Weidanz, 1970) and thus confirms effective administration of this immunosuppressive treatment in this experiment. Reduced bodyweight and survivability and feather breaks are further sequelae noted as a result of CY treatment.

The effective administration of DEX, a synthetic glucocorticosteroid, was confirmed by the significantly higher serum glucose concentration in DEX treated chickens compared to PBS2 treated chickens, as measured from Expt d21 sera. The reason for this finding, is that glucocorticoids act to increase the serum glucose concentration by stimulating hepatic gluconeogenesis (Lecocq *et al.*, 1964) and reducing glucose uptake in peripheral tissue (McKay & Cidlowski, 2003).

Given the complete absence of the APS, the effect of immunosuppression on the development of the APS cannot be determined. If the putative infectious agent/s were present in challenge material, increased susceptibility to infection leading to increased incidence of the APS could have been expected. Assuming the presence of the putative agent/s, the inability to reproduce the APS in the presence of effective immunosuppression is suggestive that the aetiology of the APS is complicated and multifactorial.

The observation of Wallerian degeneration in spinal cord segments from both Blood B and PBS challenged chickens confirms that this is an incidental finding and not induced by challenge. This is an important finding as it rules out the association of this observed pathology with the APS. Literature does not exist reporting this pathology in clinically normal broilers. One proposed reason for this observation is that the presence of Wallerian degeneration is a result of the need for rapid remodelling in the spinal cords of rapidly growing broiler chickens.

8.5 Conclusion

The APS could not be reproduced in this experiment despite immunosuppression with CY or DEX and the use of multiple batches of chickens and challenge material. Wallerian degeneration was observed in chicken spinal cords from both challenged and control chickens, confirming that the pathology is not associated with challenge or specific to the APS. One potential reason for the failure to reproduce the syndrome is the low growth rates achieved in the experiment. Further reproduction experiments should be designed to maximise broiler growth rates to enhance the chances of reproducing the APS.

Chapter 9: Advanced molecular investigations into an acute paralysis syndrome of broiler chickens

9.1 Introduction

The use of whole-genome next-generation sequencing (NGS) is becoming regarded as an alternative approach to traditional culturing and microscopy-based methods which have been used to identify new infectious agents. This has come about due to the increased availability and speed and decreased costs afforded by deep sequencing machines (Lecuit & Eliot, 2014). The technique is based on the sequence determination of all nucleic acid (NA) genomes in a sample which are then compared to sequences recorded in databases, such as GenBank, in order for the infectious agent/s to be identified. This sequencing can be performed directly from clinical samples, which subsequently includes data specific to the host genome in addition to any microbes present in the sample. The proposed advantages of NGS are that it may be faster than conventional methods, it may be more useful in identifying the agents implicated in polymicrobial infections (Hasman *et al.,* 2014) and it may allow for the screening of tissues from diseased animals in order to detect broad categories of infectious agents (e.g. viral family) that may be associated with the disease when the aetiology is unknown. Potential drawbacks include firstly, that the host genome (typically much larger than that of microbes) is also included in the output from clinical samples thus reducing sensitivity, which may require the application of strategies to increase the microbe:host NA ratio, including hydrolysis, filtration and chemical treatment in order to deplete host sequences (Barzon *et al.,* 2013). Secondly, detailed genomic information relevant to the host and microbes needs to be available (Lecuit & Eliot, 2014); however, this is less relevant when screening samples for potential microbes when the aetiology is unknown as identification at the family level can often be a useful starting point. Finally, significant computational power and expertise are required for the associated bioinformatics necessary for interpretation.

Examples of the successful application of NGS to the investigation of infectious diseases of unknown aetiology include the discovery of a novel orthobunyavirus (Schmallenberg virus) in cattle in Germany reported by Hoffmann *et al.* (2012), the recent identification of a divergent virus from the flaviviridae family associated with Theiler's disease in horses which has long been regarded as an idiopathic syndrome (Chandriani *et al.,* 2013) and the association of a novel nidovirus with the fatal neurological disease, wobbly possum disease, in Australian brushtail possums (Dunowska *et al.,* 2012). In the case of Schmallenberg virus, farmers and veterinarians

reported a syndrome in dairy cattle characterised by fever, reduced milk yield and diarrhoea from which classical microbial aetiological agents were ruled out. NGS was attempted utilising plasma from three affected cows and one clinically normal cow. Sequencing libraries were prepared separately from DNA and RNA extracted from the samples and sequences typical of orthobunyaviruses were detected in sequenced libraries from RNA in the affected animal samples and not in the sample from the normal animal. Sequence comparisons were performed using the basic local alignment search tool (BLAST) and were found to resemble other orthobunyaviruses isolated from cattle in Japan. Subsequently a reverse transcriptase (RT)-quantitative polymerase chain reaction (PCR) assay was developed to allow for screening to be performed on further field samples, essential for mitigation strategies to be implemented and to allow for further investigations to occur.

NGS thus has proven value in the early investigations into infectious diseases of unknown aetiology and thus the technique represents a potential tool for further investigation into the APS in broiler chickens, given that that it is experimentally reproducible subsequent to challenge with tissue and litter harvested from cases (Chapter 6).

There are four major steps to the process of NGS, including:

1. Library preparation
2. Cluster generation
3. Sequencing
4. Data analysis and bioinformatics (Illumina, 2014).

NGS as an infectious disease diagnostic tool requires both extracted DNA and RNA from target tissue in order to detect both DNA (bacterial and DNA viruses) and RNA (RNA viruses) genomes of infectious agents. Library preparation involves the random fragmentation of the DNA or cDNA (synthesised from RNA) sample followed by 5’ and 3’adaptor ligation. These adaptor-ligated fragments are PCR amplified and gel purified in readiness for cluster generation.

At the stage of cluster generation, the denatured fragments are loaded onto lanes on a glass flow cell which are coated with two types of oligonucleotides (oligos) which are complementary to the 5’ and 3’ adaptors attached to the library fragments. Library fragments bind to the oligos at the adaptor ends and a polymerase creates the complementary strand to each hybridised library fragment. Resultant double-stranded fragments are then denatured and the original templates are washed away. The remaining strands are clonally amplified by bridge amplification, which requires each strand to fold over and bind to the other type of oligo complementary to its free

adaptor end. A polymerase then creates a complementary strand which forms a double stranded bridge. The resultant double stranded bridge is then denatured which leaves two single stranded copies of the molecule that are tethered to the flow cell. This process of bridge amplification is repeated over-and-over again resulting in clonal amplification of all fragments in the initial library. At the end of the bridge amplification process the reverse strands are cleaved and washed off which leaves only the forward strands, of which the 3' ends are blocked to prevent unwanted priming. At this point the fragments are ready for sequencing.

The process of sequencing, termed sequencing by synthesis (SBS), utilises proprietary technology and occurs over two-reads. For each read, all clusters are sequenced simultaneously. The first read sequences the template strand whilst the second read sequences the generated complementary strand. The extension of the sequencing primer begins each read and within each read fluorescently tagged nucleotides compete for addition to the growing chain with only one being incorporated as determined by the sequence of the template. After the addition of each nucleotide the clusters are excited by a light source, resulting in a characteristic fluorescent signal being emitted and the emission wavelength and signal intensity define the base-call of the read and the number of cycles determined the length of the read. The entire SBS process generates millions of reads representative of all of the fragments.

Data analysis involves reads with similar stretches of base call being clustered and forward and reverse reads then being paired. This then creates contiguous sequences for which paired-end information is used to resolve ambiguous alignments. Further bioinformatics involves searching databases such as GenBank for known sequences similar to the sequence reads and various platforms, such as BLAST, exist in order to complete this process (Altschul *et al.*, 1990).

The purpose of the investigation set out in this chapter was to perform NGS on clinical samples from the acute paralysis syndrome (APS) affected and clinically normal sham-challenged broiler chickens in an attempt to identify a likely infectious agent(s) present in affected chickens and not in normal chickens.

9.2 Materials and methods

9.2.1 Sample preparation

Sample preparation was performed by me at the University of New England (UNE). DNA and RNA were extracted and quantified (Chapter 3) from selected tissue samples (Table 9.1). The quality of DNA and RNA extractions were assessed via gel electrophoresis using 1 % and 1.2 % agarose gels respectively and shown by the presence of a major band larger than 20 kb on the

DNA gel and the presence of two prominent ribosomal RNA (rRNA) bands on the RNA gel. Extracted nucleic acids were stored at -80 °C until ready for despatch for NGS. Extracted nucleic acids were shipped overnight (World Courier, Mascot) in liquid nitrogen from UNE to Micromon (Monash University, Melbourne) for DNA and RNA sequencing.

Table 9.1 Summary of DNA and RNA extractions for NGS

Reference	APS (Y/N)	Source	Tissue	Concentration (ng/µL)
DNA samples				
DNA 1	Yes	Field	Spleen	85.6
DNA 2	Yes	Field	Blood	105.1
DNA 3	Yes	Experimental	Blood	112.0
DNA 4	Yes	Field[1]	Spleen	14.0
DNA 5	Yes	Experimental	Spleen	666.2
DNA 6	Yes	Experimental	Spleen	32.7
DNA 7	Yes	Experimental	Spleen	28.6
DNA 8	No	Experimental - control	Spleen	414.4
DNA 9	No	Experimental - control	Spleen	177.0
DNA 10	No	Experimental - control	Spleen	266.3
DNA 11	No	Experimental - control	Spleen	264.2
DNA 12	No	Experimental - control	Spleen	146.5
DNA 13	No	Experimental - control	Spleen	412.8
RNA samples				
RNA 1	Yes	Experimental	Brain	256.1
RNA 2	Yes	Experimental	Spleen	208.0
RNA 3	Yes	Field	Spleen	318.7
RNA 4	Yes	Field	Spleen	279.4
RNA 5	Yes	Field	Spleen	346.5
RNA 6	Yes	Field	Spleen	393.9
RNA 7	No	Experimental - control	Spleen	434.5
RNA 8	No	Experimental - control	Spleen	304.2
RNA 9	No	Experimental - control	Spleen	197.1
RNA 10	No	Experimental - control	Spleen	535.5

[1]Spleen challenge material used in the experiment reported in Chapter 6.

9.2.2 Sequencing

The NGS methodology reported in this section was performed by Micromon (Monash University, Melbourne) at commercial rates.

9.2.2.1 Library preparation

DNA libraries were prepared from 100 ng of input DNA for each sample using a TruSeq Nano DNA Library Preparation Kit (Illumina Inc., San Diego) following the manufacturer's instructions. RNA was treated with RiboZero Gold (Human/Mouse/Rat) (Illumina Inc., San Diego) for the removal of ribosomal RNA (rRNA) and libraries prepared from 1 µg of total RNA for each sample using TruSeq Stranded RNA LT Library Preparation Kit (Illumina Inc., San Diego) following the manufacturer's instructions.

9.2.2.2 Cluster generation and sequencing

Cluster generation and sequencing of DNA and RNA samples were performed using a MiSeq V3 reagent kit (Illumina Inc., San Diego) in a MiSeq instrument (Illumina Inc., San Diego) following the manufacturer's guidelines producing 150 base-paired end reads.

9.2.2.3 Sequencing output quality assessment

Sequencing data output was obtained using MiSeq Reporter v2.5 (Illumina Inc., San Diego) following the manufacturer's instructions. The quality of the reads was then assessed using the software FastQC (Babraham Institute, Cambridgeshire UK).

9.2.3 Bioinformatics

Analysis of sequencing data outlined in this section was performed by Dieter Bulach (Victorian Bioinformatics Consortium and Life Science Computation Centre, University of Melbourne) at commercial rates.

9.2.3.1 Mapping of reads to reference genome sequences

Reads were mapped against the current version of the *Gallus gallus* genome (Galgal4, Ensemble Genome Browser) and the phiX174 genome using the Burrows-Wheeler Aligner (Li & Durbin, 2009). The bacteriophage phiX174 genomic DNA was spiked into samples during library preparation as a quality control measure.

9.2.3.2 Screening of reads for bacterial and viral sequences using Kraken software

Screening of reads was performed using the taxonomic sequence classification system software Kraken (John Hopkins University, Baltimore USA). This software contains a database of k-mers (short sequencing reads) which are particular to recorded bacteria and viruses. When the database is queried about a read obtained from sequencing data, it provides a label for that read which is the lowest common ancestor (LCA) based on the taxonomic information stored in the database (Wood & Salzberg, 2014). The LCA level (kingdom/order/family/genus/species/subspecies) provided depends on the level of characterisation that has been performed and subsequently added to the database for that bacterium or virus. For example, a read relating to a well characterised virus would typically be tagged to the subspecies level by the software, whereas a read relating to a novel virus may only be tagged to the level of the order or family. Reads that do not have matching k-mers in the database are left unclassified by the software (Wood & Salzberg, 2014). In the case of analysing data from biological samples, host genome sequences would typically be left unclassified by the software (Dieter Bulach, personal communication).

9.2.3.3 Further detection of viral sequences using BLAST

Following the mapping of sequence reads as set out in 9.2.3.1, reads that did not align to either of *Gallus gallus* or phiX174 reference genomes were extracted into FastQ files using SAMtools (Li *et al.*, 2009). The unaligned reads were the assembled de-novo (Zerbino & Birney, 2008) and used to construct a BLAST library which was then searched for the presence of any of the viral proteins recorded in RefSeq (National Centre for Biotechnology Information, USA). Reads which had BLAST matches of e^{-10} or greater were recorded in a spreadsheet along with their percentage identity to the matching sequence.

9.2.4 Further analysis at UNE

9.2.4.1 Bioinformatics

Further analysis of bioinformatics outlined in this section was performed by me at UNE. From the spreadsheet of BLAST matches provided by Dieter Bulach, for reads with multiple BLAST matches, the match(es) with the highest percentage identity was retained. BLAST matches from control chicken samples and equivalent matches from affected chicken samples were removed. The retained matches were then visually inspected across each of the affected chicken samples to look for evidence of a particular virus, indicated by multiple viral protein reads.

9.2.4.2 Determining the relative sensitivity and specificity of this NGS protocol

In order to determine the relative sensitivity and specificity of this NGS protocol compared to qPCR, DNA extractions previously stored (Chapter 6) from the same chicken samples used for the DNA extractions for NGS (Table 9.1) were tested for the presence and load of herpesvirus of turkeys (HVT) via quantitative PCR (qPCR), the procedure for which was described in Chapter 3. The HVT qPCR results were compared with the HVT viral protein matches from the NGS in order to calculate the sensitivity and specificity, the calculations for which are outlined below:

Sensitivity = (Number of HVT positives from NGS) / (Number of HVT positives from qPCR) x 100/1

Specificity = (Number of HVT negatives from NGS) / (Number of HVT negatives from qPCR) x 100/1

9.3 Results

9.3.1 Sequence data quality assessment and mapping to reference genomes

The read length produced from sequencing was 150 bases. The DNA reads were typically assessed to be of good quality at end-one and of usable quality at end-two. The RNA reads were of high quality at both ends.

Table 9.2 provides a summary of the reads from each of the DNA and RNA samples submitted for analysis. It shows the total number of reads sequenced (# reads), the average quality of the reads (average Phred score), the number of reads which did not map to the chicken or phiX144 bacteriophage genomes from Burrows-Wheeler alignment and this result as a percentage of the original number of reads for each sample.

Table 9.2 Summary of sequencing reads (data provided by Dieter Bulach, Victorian Bioinformatics Consortium and Life Science Computation Centre, University of Melbourne)

Reference	Number of reads	Average read quality	Number of post-mapping reads	Percentage of original
DNA				
DNA 1	483,310	32	36,653	8%
DNA 2	1,845,438	32	158,100	9%
DNA 3	227,474	32	21,389	9%
DNA 4	1,021,862	32	123,515	12%
DNA 5	1,123,162	32	134,374	12%
DNA 6	650,644	31	112,900	17%
DNA 7	896,284	31	135,932	15%
DNA 8	1,231,080	32	181,274	15%
DNA 9	719,568	31	99,553	14%
DNA 10	4,948,346	32	323,114	7%
DNA 11	8,033,428	31	1,205,546	15%
DNA 12	2,869,272	32	355,053	12%
DNA 13	1,833,116	32	214,174	12%
RNA				
RNA 1	5,012,882	35	264,100	5%
RNA 2	3,623,924	35	173,126	5%
RNA 3	4,222,232	36	146,966	3%
RNA 4	196,718	36	7,276	4%
RNA 5	396,570	36	11,678	3%
RNA 6	4,877,370	36	186,735	4%
RNA 7	16,968	35	0	0%
RNA 8	4,469,176	36	89,593	2%
RNA 9	4,488,250	36	115,917	3%
RNA 10	3,519,546	35	129,943	4%

9.3.2 Screening of reads for bacterial and viral sequences using Kraken software

Table 9.3 provides a summary of the read screening results from the Kraken software. A level of 0.2 % was set as a cut off for the labelling of matched reads, the results of which are set out in the description column of Table 9.3. Matched reads which comprised of less than 0.2 % of the total reads per samples were combined and reported as ‘other’ in the description column.

Table 9.3 Summary of read screening results from Kraken software search (data provided by Dieter Bulach, Victorian Bioinformatics Consortium and Life Science Computation Centre, University of Melbourne)

Sample	Percentage of unclassified reads	Percentage of phiX174 reads	Percentage of matched reads	Description
DNA				
DNA 1	93.91	4.85	1.24	*Clostridium perfringens* (0.22 %), other (1.02 %)
DNA 2	94.86	4.34	0.80	Other (0.8 %)
DNA 3	92.90	6.11	0.99	Other (0.99 %)
DNA 4	95.83	3.77	0.40	Other (0.40 %)
DNA 5	96.31	2.90	0.79	Other (0.79 %)
DNA 6	94.00	3.13	2.87	Felid herpesvirus 1 (0.40 %), other (2.47 %)
DNA 7	93.07	4.38	2.55	Other (2.55 %)
DNA 8	92.48	2.07	5.45	Other (5.45 %)
DNA 9	94.95	2.93	2.12	Other (2.12 %)
DNA 10	94.51	4.38	1.11	Other (1.11 %)
DNA 11	97.32	2.16	0.52	Other (0.52 %)
DNA 12	92.65	2.52	4.83	*Burkholderia* (4.24 %), other (0.59 %)
DNA 13	96.31	3.44	0.25	Other (0.25%)
RNA				
RNA 1	88.05	11.71	0.24	Other (0.24 %)
RNA 2	84.50	15.24	0.26	Other (0.26 %)
RNA 3	82.32	17.30	0.38	Other (0.38 %)
RNA 4	37.64	61.95	0.41	Other (0.41 %)
RNA 5	67.29	28.57	4.14	Gammaproteobacteria (2.04 %), Lactobacillales (0.71 %), *Clostridium perfringens* (0.30 %), *Staphylococcus spp.* (0.24 %), other (0.85 %)
RNA 6	66.56	17.68	15.76	Gammaproteobacteria (10.01 %), Acinetobacter (1.25 %), *Enterococcus spp.* (1.47 %), *Staphylococcus spp*. (0.37 %), other (2.66 %)
RNA 7	24.91	74.24	0.85	*Dichelobacter spp.* (0.24 %), other (0.61 %)
RNA 8	72.08	27.71	0.21	Other (0.21 %)
RNA 9	77.26	22.49	0.25	Other (0.25 %)
RNA 10	78.71	21.00	0.29	Other (0.29 %)

9.3.3 Further detection of viral sequences using BLAST

Figures 9.1 and 9.2 provide a summary of the total number of viral protein matches by associated virus from the affected chicken DNA and RNA samples respectively. As discussed in 9.2.4, these figures only show the viral protein matches of highest similarity retrieved from the BLAST search. Matches which were also observed in control chickens samples were removed. From the DNA samples, a total of 68 viral protein matches were found for felid herpesvirus 1 (FHV1). From the RNA samples, the largest numbers of viral protein matches were found for respiratory syncytial virus (RSV) (29 matches), CAV (18 matches) and invertebrate iridescent virus (IIV) (9 matches).

Table 9.4 provides a breakdown of the number of viral protein matches and mean percentage similarly to the recorded amino acid sequences for FHV1, CAV, IIV and RSV for each affected chicken sample.

Table 9.4 Summary of the total number of viral protein matches in samples from affected birds for the four viruses with the highest number of matches from BLAST screening

Sample	Felid herpesvirus 1 (mean similarity %)	Chicken anaemia virus (mean similarity %)	Iridescent virus (invertebrate) (mean similarity %)	Pneumovirus (Respiratory syncytial virus) (mean similarity %)
DNA samples				
DNA 1	0			
DNA 2	0			
DNA 3	2 (97.9 %)			
DNA 4	0			
DNA 5	0			
DNA 6	66 (99.6 %)			
DNA 7	1 (80.6 %)			
RNA samples				
RNA 1		4 (92.9 %)	4 (57.7 %)	0
RNA 2		1 (96.0 %)	0	0
RNA 3		10 (75.2 %)	1 (69.4 %)	0
RNA 4		0	0	11 (98.3 %)
RNA 5		0	0	5 (99.5%)
RNA 6		3 (100.0 %)	4 (59.2 %)	13 (95.1 %)

From the data shown in Table 9.4, for each virus of interest, the identification of the viral protein matches were examined for each of the positive samples. Viral protein matches consistent through all positive samples for each respective virus were only observed for CAV and RSV and the details of each of these viral protein matches is provided in Table 9.5, including the National Centre for Biotechnology Information (NCBI) database reference code for each.

Table 9.5 Consistent viral protein matches through positive samples

	Mean percentage similarity	NCBI reference
Chicken anaemia virus		
Nucleocapsid protein	99 %	NP_056775.1
Respiratory syncytial virus		
Non-structural protein 1 (1C)	100 %	NP_044589.1
Matrix protein (M)	99 %	NP_044593.1
Attachment glycoprotein (G)	97 %	NP_044595.1

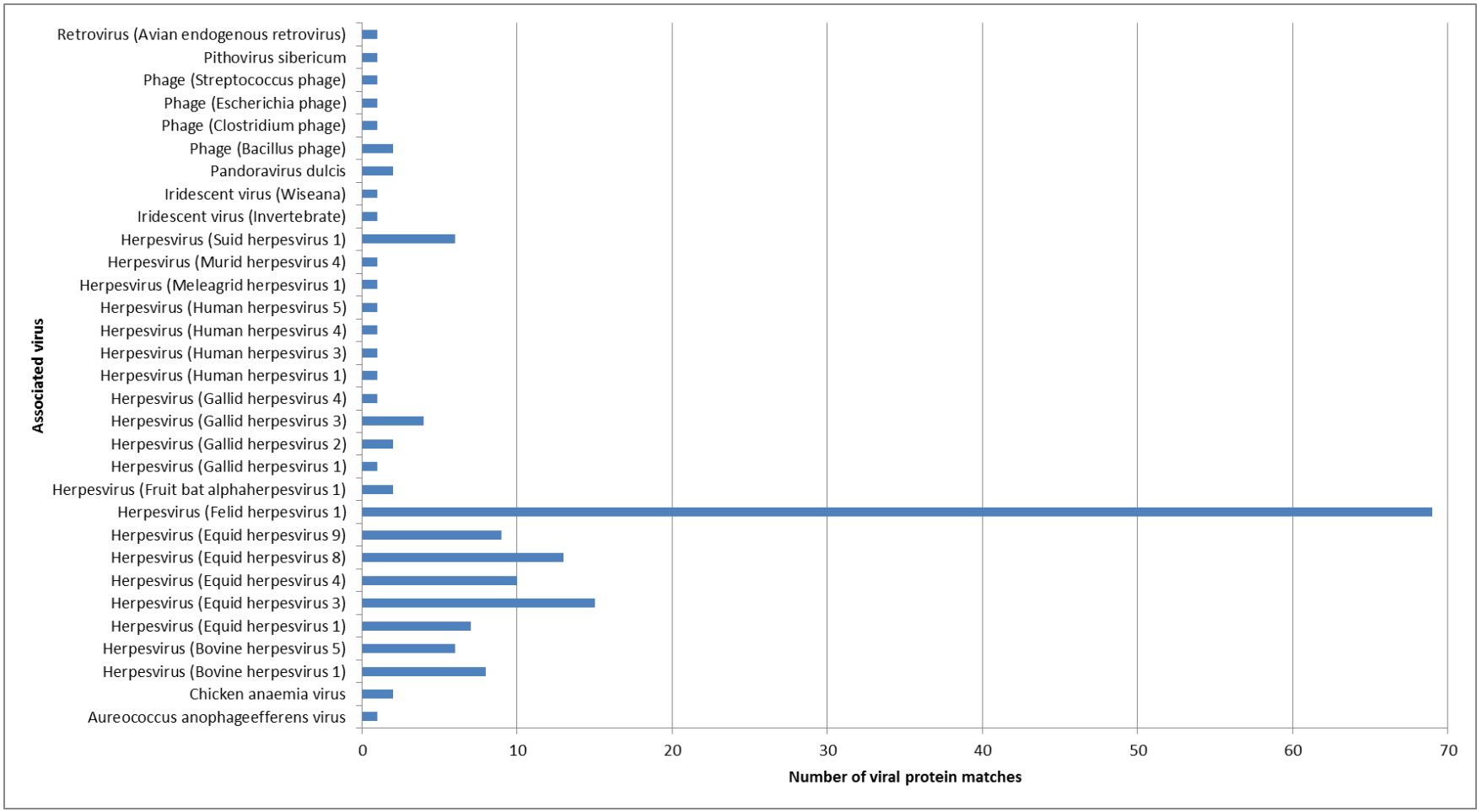

Figure 9.1 Number of matches for viral proteins from all DNA samples from affected chickens by relevant virus.

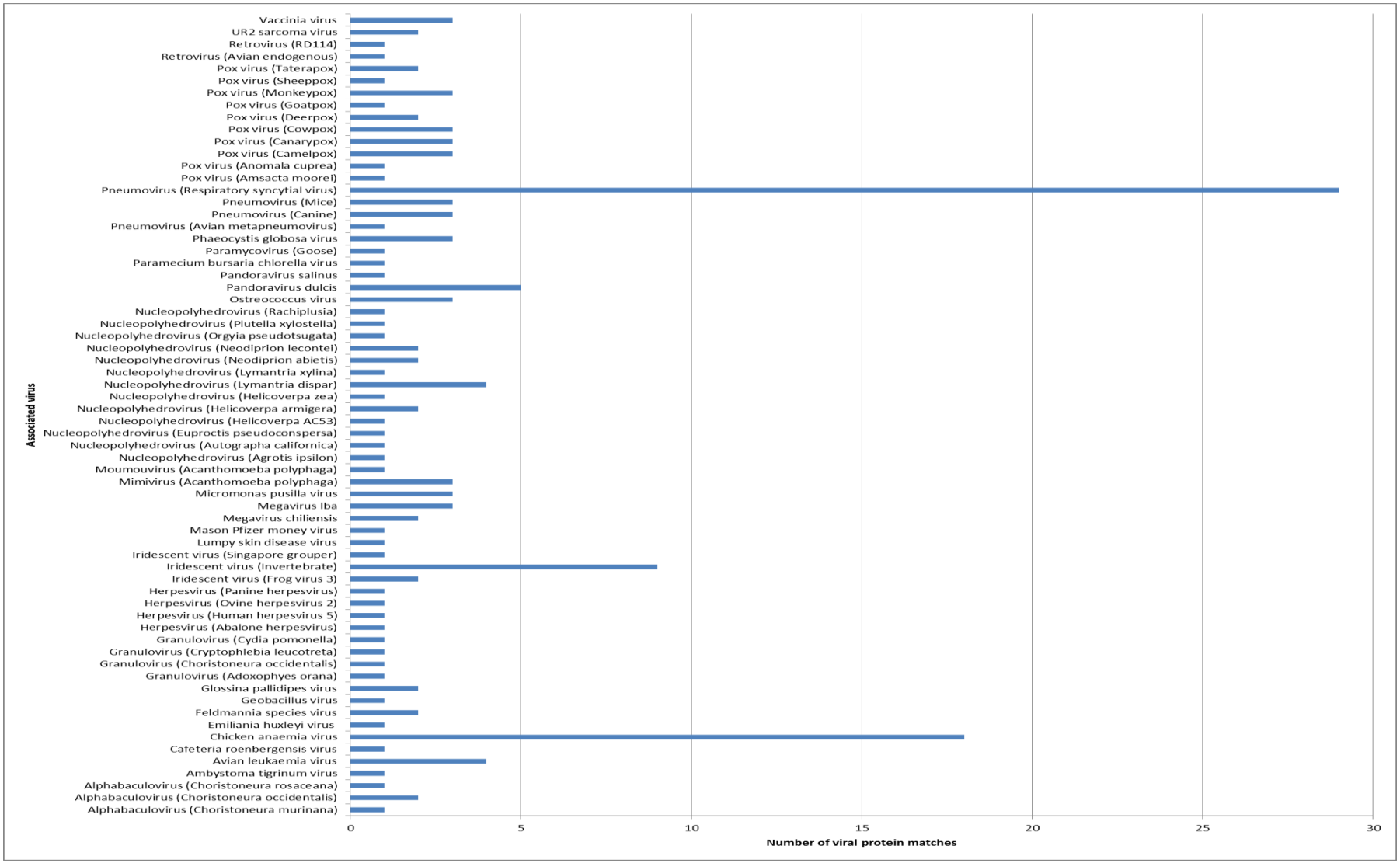

Figure 9.2 Number of matches for viral proteins from all RNA samples from affected chickens by relevant virus.

9.3.4 Determination of sensitivity and specificity of the NGS protocol

Table 9.6 provides a comparison of the NGS results and qPCR with respect to HVT. The DNA extractions used for qPCR were not the same extractions used for NGS; however, each was from the same chicken and tissue as used for NGS. The comparison outlined in Table 9.6 reveals that that the sensitivity of this NGS protocol with respect to HVT was 63 % and the specificity was 100 %. The correlation between the HVT viral protein matches and the HVT viral copy number (VCN) per reaction from qPCR was inconsistent (Table 9.6).

Table 9.6 Comparison of HVT detection ability of NGS with HVT qPCR results

Corresponding sample	HVT qPCR Result	HVT VCN per reaction (25 ng/µL of DNA)	NGS Result	HVT viral protein matches
DNA 1	n/a	n/a	Negative	0
DNA 2	n/a	n/a	Negative	0
DNA 3	Positive	243,692	Negative	0
DNA 4	Positive	2,498,944	Positive	1
DNA 5	Positive	26,568	Positive	2
DNA 6	Positive	726,423	Positive	7
DNA 7	Positive	126,268	Negative	0
DNA 8	Positive	42,916	Negative	0
DNA 9	Negative	0	Negative	0
DNA 10	Positive	414,732	Positive	5
DNA 11	Negative	0	Negative	0
DNA 12	Positive	26,894	Positive	2
DNA 13	Negative	0	Negative	0

HVT = herpesvirus of Turkeys, NGS = next generation sequencing, PCR = polymerase chain reaction, VCN = viral copy number.

9.4 Discussion

The DNA was typically found to be of good quality at end-one and of reduced but useable quality at end-two. The lower quality of end-two reads may have been due to the large size of DNA fragments in the biological samples which meant that the fragmentation of DNA may not have been sufficient to produce the required DNA fragment size (Dieter Bulach, personal communication). RNA samples were of good quality at both ends.

The summary of sequencing reads provided in Table 9.2 shows that sequencing reads were of high accuracy as the average quality of each read exceeded 30, which is associated with 99.9 % base call accuracy or the probability of a base call being incorrect at 1 in 1,000 bases using the Phred scoring system (Ewing & Green, 1998). There was an obvious problem with the sample RNA 7 as Burrows-Wheeler alignment reported nil post-mapping reads (Table 9.2) and the Kraken screening of the sample found that 74.24 % of sequenced reads were associated with the phiX174 genome (Table 9.3) which was spiked into all the samples initially. The sample RNA 4 also had a high percentage (61.95 %) (Table 9.3) of sequenced reads associated with the phiX174

genome; however, Burrows-Wheeler alignment reported 4 % post-mapping reads which was comparable with most of the other samples (Table 9.3).

The Kraken software was the only method used to identify bacterial sequences in the samples. The software also detects viral sequences. However, to increase the sensitivity in detecting evidence of viral sequences in the assembled unaligned reads, BLAST was also used as the APS appeared to be more similar to other viral diseases of poultry as opposed to bacterial diseases (Chapter 2, 4). Kraken has been shown to have high classification accuracy, with its classification sensitivity (proportion of sequences assigned to the correct genus) exceeding 90 % and its classification precision (proportion of correct classifications out of the total number of classifications attempted) 99.9 % (Wood & Salzburg, 2014). Thus, it was felt that if a bacterium was present in any of the samples we could be confident that it would be detected. The relative sensitivity of the BLAST technique compared to qPCR with respect to the detection of HVT was 63 % and the relative specificity was 100 %. From these results, we can be relatively confident that false positives were not detected from the NGS; however, there was a moderate chance of false negatives being associated with the NGS results.

Evidence of *Clostridium perfringens* was detected in DNA 1, which was DNA extracted from the spleen of an affected field chicken. This most likely represents a contaminant from sampling as *C. perfringens* is regarded as normal flora of the chicken gastrointestinal tract (GIT). Evidence of bacteria of genus *Burkholderia* was detected in DNA 12. *Burkholderia* is a genus of Proteobacteria within class Betaproteobacteria (NCBI, 2015). This DNA sample was extracted from the spleen of a sham-challenged experimental broiler chicken (Chapter 6) and thus is not associated with the APS. It most likely represents a PM sampling contaminant.

From the RNA samples, NA sequences of bacterial origin were found in two of the six affected chicken samples, RNA 5 and RNA 6. NA sequences typical of Gammaproteobacteria (a class of Proteobacteria) (NCBI, 2015) were detected in both samples. Common species of bacteria within this class include *Salmonella spp.*, *Escherichia coli* and *Pseudomonas spp*. Given that they were only detected in two of the six affected chicken samples it likely represents a PM sampling contaminant. NA sequences of *Staphylococcus spp.* origin were also detected in both of the samples and these were also likely a sampling contaminant. In the sample RNA 5 there was evidence of *C. perfringens* contamination as discussed above. In this sample there was also evidence of NA sequences of Lactobacillales origin. Lactobacillales is an order of the class Bacillus which includes the common environmental microbial *Streptococcus sp.* and the GIT microbial *Lactobacillus sp*. (NCBI, 2015) and thus is also likely a PM contaminant. In the sample

RNA 6 there was evidence of NA sequences of Acinetobacter and *Enterococcus sp.* origin. The former is a genus of Proteobacteria (class Gammaproteobacteria) (NCBI, 2015) and bacterial species' in this genus are common soil microbes and thus represents a likely contaminant. *Enterococcus sp.* is commonly a GIT commensal organism and thus in this case also likely represents a PM sampling contaminant.

NA sequences of *Dichelobacter sp.* origin were detected in the RNA sample RNA 7. As it was only detected in one sample it is unlikely to be relevant to the APS. Of interest however is that the laboratory in which the NGS was performed has worked with *Dichelobacter nodosus* in the past which raises the possibility that this finding in RNA 7 was a laboratory contaminant.

NA sequences with high similarity to FHV1 were detected in the DNA sample DNA 6. This finding was further supported by a large number of viral protein matches to FHV1 being detected in the sample from the BLAST search. In relation to the latter, these matches were of high sequence similarity (Table 9.4). FHV1 is an alphaherpesvirus (Maes, 2012), as are MDV and infectious laryngotracheitis virus of poultry. There is no literature available to confirm that avian species sit within the host-range of FHV. However, this idea is not inconceivable given that feline pests are commonly seen around commercial poultry facilities in Australia; thus, exposure to FHV would have to be considered likely. The presence of FHV1 in only one of the seven affected chicken DNA samples suggest the association of FHV1 to the APS is unlikely; however, not impossible given that the sensitivity of the BLAST used in this case was only moderate as earlier discussed. Further screening of a larger number of samples would be a logical next step using PCR, of which there are a number of reported assays in the literature (Persico *et al.*, 2011; Sandmeyer *et al.*, 2010).

Low numbers of viral protein matches to CAV were detected in four of the six affected chicken RNA samples. This is not surprising given the serological results obtained from the experiment reported in Chapter 6 in which control chickens were all seronegative for CAV, whereas 30 % of affected broilers tested were CAV seropositive (Table 6.11). The serological results reported in Chapter 6 indicated a level of transfer of the virus through the challenge materials. Given the likelihood of infection with CAV, it would have been expected that more matches to CAV viral proteins were found. The most likely explanation for this was that the CAV was in the splenic tissue of affected chickens at the time of sampling in low numbers, consistent with the kraken search not detecting CAV and work by Joiner *et al.* (2005), who reported that inoculated experimental broilers carried low levels of the virus.

A small number of viral protein matches with high similarity to IIV were found in the one brain RNA sample analysed and in two of the five affected chicken spleen RNA samples analysed. Iridescent viruses are most associated with fish from a veterinary disease perspective, such as lymphocystis disease, which is characterised by nodular growth on the fins, skin and gills of infected fish (Hess, 1981). Disease in avian species caused by iridescent viruses has not been reported, thus the presence of similar viruses in this case is interesting but not useful at this stage given that a consistent viral protein match was not observed in each of the positive samples, which would make the design of a PCR reaction to further screen samples difficult. Given that the kraken search did not detect IIV, any virus present would likely be so in low numbers.

Matches of high similarity were detected to RSV in three of the five RNA samples from affected chicken spleen samples and not in the affected chicken brain sample. RSV belongs to the family *Paramyxoviridae,* in which also sit avian metapneumovirus (aMPV). The latter is known to cause mild respiratory disease and less commonly swollen head syndrome in chickens (Gough & Jones, 2008). There is not a significant body of literature supporting disease in chickens specifically from RSV. Interestingly the matches found in the three samples had a high level of similarly to RSV and not aMPV. As RSV is a virus causing respiratory disease in humans, it is conceivable that RSV is a human contaminant from sampling and processing of the chicken tissue. Given that viral protein matches to RSV were detected in 60 % of the affected chicken spleen RNA samples this finding is potentially of interest to the APS, particularly given that the NGS technique employed only demonstrated moderate sensitivity relative to qPCR. It is typically expected that large numbers of viral protein matches to RSV are observed in samples from tissues infected with reasonable quantities of virus (Dieter Bulach, personal communication). However, we observed a poor correlation between the derived VCN from qPCR and viral protein matches from NGS with respect to HVT. Nevertheless, given that the kraken software did not detect RSV, its presence in tissue would likely be in low numbers. Using sequence data specific to the viral protein matches through all positive samples (Table 9.5), an RT-PCR could be developed to test additional samples. Based on the results presented in Table 9.5, all matches to RSV non-structural protein 1 (1C) were 100 % identical to the recorded sequence. Thus, this would be a logical sequence on which to design an RT- PCR test.

9.5 Conclusion

NA sequences of FHV1 origin were detected in one of seven DNA samples from chickens affected of the APS. NA sequences of RSV origin were detected in three of five RNA samples from splenic tissue of chickens affected with the APS. These findings may be of interest for further investigations into the APS. Performing PCR tests specific to each of these viruses would allow for the rapid screening of affected chickens from further cases and is the next logical step following from these investigations.

Chapter 10: General discussion and conclusions

The primary purpose of the research undertaken and reported in this thesis was to determine the aetiology of the acute paralysis syndrome (APS), risk factors for it and management for controlling it. The approach taken was firstly, to define the APS based on the clinical syndrome and diagnostic pathology and testing performed and in doing so devise a differential diagnosis list to direct further investigation. Secondly, through detailed epidemiological investigation, to identify risk factors associated with the presence of the APS in broiler flocks. Finally through experimental investigation to define a reliable experimental model for reproducing the APS and further develop and refine the differential diagnosis list.

Although a definitive diagnosis for the APS was not determined, the overarching objectives of the research were met. The findings from this research provide a suitable basis for further investigation into the causation of the APS. Comprehensive discussion has been provided within relevant chapters of this thesis. This section will review the main findings and discuss further implications of this work.

10.1 Background to the APS (Chapter 4)

The most striking features of the APS were the marked flaccid paralysis observed associated with the neck of affected chickens (Chapter 4) and the associated spike in flock mortality (Chapter 5). Early diagnostic testing firstly ruled out the notifiable diseases Newcastle disease (ND) and avian influenza (AI), both of which can have associated neurological syndromes; however, with obvious differences to the APS (Chapter 2). At this point, diagnostic testing focussed on the two most similar disease syndromes based on clinical appearance; botulism and the acute transient paralysis (TP) form of Marek's disease (MD). Botulism induces flaccid paralysis of the neck; however, this paralysis typically also includes the head, legs and wings which is an obvious point of difference to the APS. Diagnostic testing to definitively rule out botulism is difficult. Although the mouse inoculation bioassay is widely regarded as the most sensitive method to diagnose botulism (Dohms, 1987; Thomas, 1991), this approach was not used. Instead a botulinum toxin ELISA was used, which is considered to be less sensitive than the mouse inoculation bioassay (Thomas, 1991). However, given the obvious difference in the APS and botulism with the lack of paralysis associated with the eyelids, wings and legs in the former, the CNS histopathology associated with the APS, in conjunction with the negative botulinum toxin ELISA results, it was felt that *Clostridium botulinum* was an unlikely cause and could be justifiably ruled out.

The acute TP form of MD reported in field (Kenzy *et al.,* 1973; Zander, 1959) and experimental chickens (Swayne *et al.,* 1989abc; Witter *et al.,* 1999) is similar to the clinical appearance of the APS and the histopathology observed in the brain, being the presence of vasculocentric encephalitis focussed in the cerebrum for the APS and cerebellum for the TP form of MD. These similarities in conjunction with high Marek's disease virus serotype-1 (MDV1) load in shed dust (Chapter 4) lead to a tentative diagnosis of MD being made, which was further supported by the detection of MDV1 in spleen and brain tissue from affected chickens reported in Chapter 4 and Wajid (2013) also detecting MDV1 in spleen tissue from chickens affected with the APS. However, the implementation of herpesvirus of turkeys (HVT) vaccination of broilers did not bring about a reduction in the incidence of the APS. This could be explained if the APS was not caused by MDV1 or if the causative MDV1 was sufficiently different from other MDVs that HVT did not induce protection. Cases in which HVT has not provided protection against MDV1 are reported in the literature (Eidson *et al.,* 1981; Witter *et al.,* 1980). However HVT has been shown to be fully or partially protective against Australian strains of MDV (Renz *et al.,* 2012; Walkden-Brown *et al.,* 2013) and vaccination in this case was followed by significant reductions in the MDV1 load in shed dust samples suggestive of HVT efficacy. This result was therefore inconsistent with the possibility that HVT did not provide protection against the causative MDV1. Nevertheless, following the introduction of HVT vaccination MDV1 was detected in the tissues (spleen and brain) from a small proportion of HVT vaccinated and APS affected chickens. Given this, MDV1 was considered unlikely to be the cause of the APS but could not be ruled out at the commencement of the experimental and diagnostic investigations reported in this thesis. Hence early experimental reproduction efforts focussed on definitively determining whether MDV1 was involved as an aetiological agent of the APS (Chapter 6).

The inflammatory histopathology observed in the brains of a proportion of chickens affected with the APS was most similar to viral infections of poultry including the TP form of MD (discussed above), ND, AI, avian encephalomyelitis (AE) and arboviral disease (such as West Nile virus infection) (Chapter 2, Chapter 4). Early diagnostic investigations ruled out AI and ND. Affected chickens were vaccinated against ND but encephalitis induced by the ND vaccine as reported by Nakamura *et al.* (2008), was ruled out as a cause of the APS given the marked differences in the clinical syndromes between the APS and that reported by Nakamura *et al.* (2008). There had not been any diagnostic work performed at the commencement of this research work pertaining to the potential for AE virus or arboviruses as aetiological agents of the APS, which provided direction for further investigation.

Although the histopathology associated with the AE has similarities with the APS, the neurological syndrome associated with AE is characterised by depression, ataxia and muscle tremors in young chickens, typically within the first 2 weeks of life (Calnek, 2008), which is distinctly different to the APS. Neurological disease associated with AE is considered rare in chickens older than 3-4 weeks of age (Calnek, 2008). Thus if AE virus was identified as an aetiological agent associated with the APS, it would be an atypical form of AE.

Neurological disease caused by arboviruses has not been reported in poultry in Australia. However, recently a neurological syndrome has been observed in equine species in Australia caused by Kunjin virus, a West Nile virus (WNV) subtype and member of the *Flaviviridae* family. Given the presence of Kunjin virus in Australia and the presence of a mosquito vector in the region affected by the APS, interest developed to test for the presence of flaviviruses in affected chickens.

It was considered unusual that there was microscopic evidence of degenerative pathology in the spinal cords of APS affected birds given that spinal reflexes were intact. As the sensory pathways within the spinal cord are located in the outermost regions and are required for spinal reflexes to be intact, reflexes should be abnormal in the presence of significant degenerative pathology in these regions. This raised the possibility that the spinal cord pathology was incidental and unrelated to the syndrome.

The collation of historical information associated with the APS as reported in Chapter 4 provided a clear rationale for further and more directed investigations, as reported in Chapters 5-9.

10.2 Epidemiological findings associated with the APS (Chapter 5)

The APS resulted in in significant elevations in flock wastage (mortality and culls) and is thus of economic and welfare concern to the producer. Cases of the APS were predominantly reported on two farms which contained large modern sheds capable of holding large numbers of chickens. For the epidemiological analyses to be useful we needed to identify the factors associated with these two farms that increased the risk of the APS developing. Logistic regression analysis was considered the ideal multivariate model to be used for this as the outcome variable, the presence or absence of the APS, was binary. The logistic regression model required the continuous variables, such as temperatures and bodyweights, to be broken down into meaningful units (Hosmer & Lemeshow, 2000). It was decided that taking one standard deviation of the dataset for continuous variables as the unit was the best approach in this case as it was a simple, consistent

and unbiased approach. Of additional benefit is that multivariate logistic regression models acts to adjust for confounding factors (McNamee, 2005) which were likely in this case given the relative isolation of cases of the APS to two farms with the largest flock sizes and most modern sheds. The main limitation associated with these analyses was that the final model may not be an accurate representation of the field situation as ideally a dataset covering a longer period of time would be utilised. Thus factors identified to be associated with the APS from univariate analyses that did not persist in the final logistic regression model cannot be ignored.

The epidemiological analyses revealed a clear trend for the APS to be more prevalent in flocks placed in spring and summer. This finding is consistent with associations found between higher shed and regional temperatures and the presence of the APS. Heat stress can increase the susceptibility of chickens to a range of diseases as it impairs immunological functions, affecting macrophage function and also decreasing relative mass of the bursa of Fabricius, thymus and spleen (Quinteiro-Filho *et al.,* 2010). An obvious trend of increased prevalence of the APS could be expected if the aetiological agent was an arbovirus as mosquitos are much more prevalent at warmer times of the year in the region affected by the APS. However, flaviviruses which were deemed to be the most plausible arbovirus implicated in the APS were not detected in affected chickens (Chapter 6).

The finding that the APS was more prevalent in sheds of new design was interesting; however, this required caution in interpretation as new design sheds were only present on the two farms which accounted for the majority of cases of the APS, leading to complete confounding of the effects of shed design and farm. In the period 26 - 51 days of age during which maximum shed temperatures were significantly higher in flocks affected with the APS, the shed temperature difference between new and old sheds was not significant; however, the fluctuation in shed temperature was significantly greater in new than old design sheds. This raises the question as to whether the modern ventilation systems in new sheds result in chickens being cooled too quickly when heat stressed. This provides some ground for further investigation. Bodyweights recorded at all ages were significantly heavier in the new compared to old sheds. This could explain why the APS was found to be more prevalent in flocks placed in new design sheds given the significantly greater odds of the APS developing in heavier flocks reported in the logistic regression analyses. However, there was no significant interaction between the effects of the APS (present/absent) and shed design on bodyweight at any measurement age suggesting that the effect of bodyweight was independent of shed design. It is to be expected that bodyweights are higher in new design sheds given ventilation/climate control and feed distribution technologies.

There was a significantly greater prevalence of the APS in larger flocks. There was potential for confounding of this result because the largest flock sizes were placed on farms 13 and 14 which accounted for the majority of cases of the APS. It was felt that flock size as a risk factor could be important, for example larger flock sizes provide greater number of hosts for an infectious agent to propagate in which may lead to faster onset of clinical disease in a population with a short life-span as is the case for broiler chickens. The importance of flock size as a risk factor was further highlighted in the multivariate logistic regression analysis, supporting this idea. As a mitigation strategy flock size as a risk factor is not useful as it was found to be independent of stocking density (consistent across all flock sizes) and farms could not economically justify reducing flock sizes utilising current infrastructure.

Better performing flocks as determined by bodyweights were at greater risk of developing the APS. This risk was significant at 14, 21 and 28 days of age over all flocks. However, the risk in better performing males in sexed flocks was significant at all ages except 7 days. It was observed that male but not female mortality was significantly greater in sexed flocks with the APS than sexed flocks without the APS. This suggested that males were more susceptible to the APS than were females, which was later confirmed from experimental findings (Chapter 6) and such may be linked to growth rates. The apparent increased susceptibility of better performing broiler chickens to the APS is somewhat similar to broiler ascites syndrome for which there is often believed to be a positive correlation with high bodyweight (Crespo & Shivaprasad, 2008). In ruminants, enterotoxaemia from *Clostridium perfringens* type D, which is considered normal intestinal flora, is more common in faster growing animals (Michelson & Smith, 2009). However for most infectious veterinary diseases, better performing animals are typically more resistant. This doctoral project did not investigate the possibility of enterotoxaemia as a cause of the APS as the APS does not fit the clinical and neuropathological syndrome of enteroxaemia and it occurs in ruminants and enterotoxaemia is typically not reported in poultry. Despite these reasons, further investigation with respect to this possibility may be justified. Furthermore, it may be justified for future work to investigate any protective effects of early growth restriction in relation to the APS. Univariate analysis showed the highest odds of the APS developing to be associated with increases in 14 day of age bodyweight above the Ross breed standard guidelines, thus further investigations should focus at least on growth restriction within the first 14 days of age.

The association between the presence of the APS and higher bodyweights seems to be contradictory to the association between APS and below standard maximum brooding temperatures. It would be expected that if brooding conditions were inadequate in the first week of life that subsequent performance would be affected. This cannot be explained from the dataset

and highlights the need for epidemiological analyses to be performed on a dataset covering a longer time period and also multiple production regions. The results of logistic regression analysis revealed a high level of risk of the APS from below standard maximum brooding temperatures in the first week of life. Thus tighter brooding temperature monitoring and controls should be put into place to reduce such risk. There is a link between broiler ascites syndrome and cold temperatures; however, in the case of broiler ascites the sub-optimum environmental temperatures are not isolated to the first week of life (Crespo & Shivaprasad, 2008; Pakdel *et al.*, 2005).

Analysis of maximum shed temperatures showed a significant difference between flocks with and without the APS in the period 26 - 51 days of age encompassing the majority of APS cases, but not in the period 0 - 25 days of age prior to onset of most cases of APS. This was investigated further through logistic regression analysis of factors influencing APS and this revealed that the highest risk of developing the APS was from elevations in shed temperature above the Ross 308 standards in the period 29 - 35 days of age. This period represents the commencement of the 2nd and most significant spike in mortalities in flocks with the APS. These findings are consistent with seasonal variation in prevalence of the APS and highlight that tighter monitoring and control of maximum shed temperatures at warmer times of the year are a necessary strategy to reduce the incidence of the APS.

Flocks with the APS experienced significantly greater shed temperature fluctuations throughout their lives than did flocks without the APS. Consistent with this, shed temperature fluctuations were greatest in new as opposed to old design sheds. The most plausible reason for the finding in relation to shed design is that newer sheds which have installed modern ventilation systems and are of a more thermostable design, can be cooled much more effectively and quickly when shed temperatures increase. Whilst it would be expected that new design sheds should not reach as high maximum shed temperatures at warmer times than old sheds. It may be possible that in the event of new sheds reaching higher than ideal maximum shed temperatures, management interventions may correct high shed temperatures too quickly. This relates to earlier discussion and provides grounds for future investigation.

High maximum daily external temperature and fluctuations in external temperature were risk factors for the APS. These findings are consistent with a higher prevalence of the APS in flocks placed at warmer times of the year and also with shed temperature findings as previously discussed. As external temperature cannot be controlled via management intervention, these findings highlight that external temperature monitoring could be an important strategy in reducing

the incidence of the APS. For example, ventilation changes could be put in place early enough to prevent or reduce the levels of heat stress experienced by chickens.

Significant risk of the APS developing was found to be associated with delays in the first flock thin out. Delays in thinning-out likely increases the stress placed on chickens associated with stocking densities, such as elevated shed temperatures and access to feed and water. The mean 1st thin-out time for flocks with the APS was 35.14 ± 0.22 days and for flocks without the APS 34.62 ± 0.06 days. It is thus recommended that the 1st-thin out of a flock occurs prior to the 35th day of age.

From the multivariate logistic regression model, three significant risk factors for the APS were identified. In order of decreasing level of risk these were mean daily maximum shed temperature below the Ross 308 standards in the first week of life, increased flock size and increased mean daily maximum regional temperatures through the life of the flock. Additionally there was significant interaction between the effects of mean daily maximum regional temperature and mean daily maximum shed temperature in the period 15 - 21 days of age such that increases in regional temperature were associated with sharper increases in maximum shed temperature in flocks with the APS in the period 15-21 days of age. This analysis indicates that in order to reduce the incidence of the APS the following steps should be implemented:

- Aim for brooding temperatures in the first week of life to not fall below the level of the Ross 308 breed standards;
- Monitor weather forecasts and record external temperatures so that times of highest risk of the APS can be managed; and
- Review ventilation and cooling strategies for warmer times of the year particularly on farms 13 and 14 with particular focus on the period 15 - 21 days of age. The goal should be to reduce impact of high external temperatures on shed temperatures and limit the extent of temperature fluctuations in the sheds.

10.3 Initial experimental reproduction of the APS (Chapter 6)

The development of an experimental reproduction model is a key component of investigations into disease causation, enabling the testing of specific hypotheses or predictions under controlled conditions. The key objectives realised from the experiment reported in Chapter 6 (13-C-REP1) were firstly to define this experimental reproduction model and secondly, to determine that the APS was transmissible, and thus likely to have an infectious aetiology. Chapter 6 reported the successful experimental reproduction of the APS in broiler chickens challenged with spleen cells

or whole blood at 21 days of age or exposed to litter from affected flocks from 21 days of age. These results strongly support the involvement of an infectious agent/s present in lymphoid cells and also potentially in a free state in blood. The agent/s is also shed from the affected chickens such that it contaminates litter, persisting sufficiently to transmit to chickens placed on that litter. Examples of routes for shedding could be via the feather follicular epithelium, as occurs in the case of Marek's disease, or via the faeces, as occurs in a range of viral infections of poultry.

The inability to reproduce the APS in broiler chickens challenged at day-old is also consistent with an infectious aetiology of the APS, based on the assumption that these chickens would be protected against the agent/s by maternally derived immunity. Furthermore, it is consistent with the observed timing of the APS in the field. Maternal immunity typically provides protection to chickens against infectious challenge in the first 10 - 14 days of life (Patterson *et al.,* 1962). Thus such maternal immunity would likely have decayed to a non-protective level for the chickens challenged at 21 days of age, in which the APS was reproduced. Given the pattern of reproduction in broilers, the inability to reproduce the APS in any specific pathogen free (SPF) chickens free of maternal antibodies to a range of infectious agents was surprising. This is suggestive that susceptibility of chickens to the APS is genotype specific. There have been anecdotal reports of similar syndromes in Ross broilers in New Zealand and the United Kingdom, based on discussions when these results have been presented at conferences (Page xiii). There have been no such reports of the APS in other breeds of broiler chicken, such as the Cobb 500. Genetic variation in susceptibility to infectious diseases in poultry is reported, including for MD (Sharma & Stone, 1972), infectious bursal disease (IBD) (Bumstead *et al.,* 1993) and *Salmonella typhimurium* (Bumstead & Barrow, 1988). The APS has not been reported in breeding or laying stock in the affected region and other broiler breeds are not grown in the affected region. The experimental results reported in Chapter 6 and anecdotal reports received suggest that the Ross 308 broiler chicken is inherently susceptible to the APS. However, the producer does grow Ross 308 broiler chickens in other regions in Australia where the APS has not been reported.

Male broiler chickens were found to be more susceptible to the APS than were females. This finding is consistent with epidemiological findings which showed that male but not female mortality increased significantly in sexed flocks with the APS (Chapter 5). Two reasons for the increased male susceptibility can be reasonably postulated. These are the immunosuppressive role of male androgens and their effects on growth rate. Male androgens are known to have a suppressive effect on male immunity in humans (Klein, 2000). Consistent with this, in chickens, Chen *et al.* (2010) found that caponisation of male chickens resulted in significantly greater relative bursal weight and antibody titre post ND virus challenge. The alternative postulate is that

the APS, being more associated with faster growing flocks (Chapter 5), has a greater effect on males, as they grow at a faster rate than females (Aviagen, 2012). Infectious disease is typically more associated with reduced growth rates in animal populations rather than faster growth rates. Thus this theory in relation to the APS represents a unique scenario. As was discussed in Chapter 5, enterotoxaemia is an example of clinical disease induced by the presence of the infectious agent being more associated with faster growing animals. A point of difference here with respect to the APS, is that *Clostridium perfringens* is normal flora of the gastrointestinal tract (GIT) and certain conditions favour its over-proliferation leading to enterotoxaemia. Given the failure to reproduce the APS in experimental control birds, it would be unlikely that the agent/s is normally present in broiler chickens. Furthermore, it would be unlikely that *C. perfringens* would be transmitted via lymphocytes and blood as was demonstrated in this experiment for the APS.

MDV1 as a cause of the APS was conclusively ruled out from the experiment reported in Chapter 6. This was the case given that MDV1 could not be detected in tissues (spleen and brain) from chickens in which the APS was reproduced and additionally supported by the finding that HVT did not afford protection against the APS in broiler chickens in the experiment. By running three separate polymerase chain reactions (PCRs) (two different standard PCRs and one quantitative PCR), in which the primer sets targeted different regions of the MDV1 genome, the risk of missing any MDV1 present due to genetic variation of the virus was minimised.

Vasculocentric encephalitis was observed in brain sections from all chickens which demonstrated the APS in this experiment. The focus of this pathology was at the base of the cerebrum. The neurological examination findings from experimental chickens affected with the APS were also consistent with affected field chickens, localising neurological deficits to within the forebrain. With the location of visible pathology in the brains of affected chickens being consistent with deficit location from neurological examination findings, the brain pathology was likely associated with the observed clinical signs in affected chickens. This pathology was only observed in 32% of samples submitted from affected field chickens (Chapter 4). The most plausible reason for a higher rate of detection of the pathology in experimental chickens was that tissue fixation occurred immediately following euthanasia in the experiment (except for three chickens which were submitted chilled). In field cases, immediate fixation of samples was often not achieved (Rod Reece, personal communication). Improved fixation of tissues would have enhanced the ability to detect lesions as post-mortem degeneration could have disguised any pathology present, particularly if subtle. Additionally, all experimental samples were analysed by Rod Reece, a senior avian pathologist at the State Veterinary Diagnostic Laboratory (SVDL). Field samples

were analysed by a range of pathologists, depending on who was allocated to each submission at the time. Thus a more consistent set of results could be expected from the experimental samples.

Histological examination of spinal cords was only performed on three affected chickens from this experiment, each of which was submitted as a chilled whole dead bird to the SVDL. Wallerian degeneration in the spinal cord was observed in two of these chickens. Vasculocentric inflammatory pathology was observed in the spinal cords from two of these three chickens also. Fixed spinal cord samples were not stored and submitted from the remainder of experimental chickens affected with the APS because spinal cord pathology was not considered to be a major feature associated with field cases at the time. Except for in one chicken, degenerative and/or inflammatory spinal cord pathology had not been reported by the SVDL (Chapter 4) prior to the operation of this experiment. Vasculocentric inflammatory pathology was not reported by the SVDL in the spinal cord sections submitted from the field (Chapter 4). The presence of spinal cord pathology was not consistent with neurological examination results, which localised the site of neurological deficit to within the forebrain. It would be expected that there would be spinal reflex deficits with the observed spinal cord pathology. This raised the question as to whether the observations within the spinal cord were actually incidental.

In human and veterinary patients, encephalitis can be infectious or non-infectious in aetiology. Infectious aetiology can be viral, bacterial, parasitic or protozoal. Auto-immune encephalitis is the predominant non-infectious aetiology of encephalitis in humans. Viruses are generally considered to be the most common cause of encephalitis in human and veterinary populations. In humans in the USA, herpes simplex virus type 1 (HSV1) is the most common cause of encephalitis (Corboy & Tyler, 2004). However, epidemics of human encephalitis caused by arboviruses do also commonly occur (Corboy & Tyler, 2004). If an infectious agent induced the vasculocentric encephalitis observed in chickens affected with the APS it would be expected that the agent would be visibly associated with CNS lesions. Histopathological examination of field (Chapter 4) and experimental (Chapter 6) chickens affected with the APS has repeatedly failed to detect evidence of bacterial colonies, protozoa or other parasites, largely ruling each of these out as aetiological agents associated with the APS. Electron microscopic evaluation of a selection of brain sections from experimental chickens failed to detect the presence of viruses or evidence of viral infection, such as cell apoptosis, which could implicate a virus as the cause of the encephalitis in chickens affected with the APS. In cases of cerebral encephalitis of viral origin, viral particles can often be seen in capillary endothelial cells, as is often the case in humans from infection with picornaviruses (e.g. poliovirus), togaviruses, bunyaviruses, reoviruses and retroviruses (Corboy & Tyler, 2004). Literature reporting the sensitivity of electron microscopy as a diagnostic tool in

cases of viral encephalitis in animals is limited. In human cases of culture-positive HSV1 encephalitis, White & Taxy (1983) reported a sensitivity of only 56% for electron microscopy in detecting viral particles in brain tissue. Thus for the APS, viral encephalitis cannot be ruled out completely based on the absence of virus in electron microscopy sections.

AE (a picornavirus) was considered as a differential diagnosis for the APS given that it is a neurological syndrome affecting poultry and is associated with somewhat similar brain pathology to the APS (Chapter 2, Chapter 4). Furthermore 40% of broilers affected with the APS were AE virus seropositive in 13-C-REP1. Diagnostic investigations into field cases of the APS had not considered the potential involvement of AE virus in the APS at this point. In the reproduction experiment varying proportions of terminal broiler sera from challenged groups were seropositive to AE virus and all control samples were seronegative, which suggested that AE virus was transferred via the challenge material. However, only one SPF chicken was seropositive to AE virus. Vertical transmission of AE virus is an important and well recognised mode of transmission (Calnek, 2008; Van Roekel *et al.,* 1941) and thus given the serological results from SPF chickens, it is highly likely that a level of vertical transmission of the virus occurred in the broiler chickens. AE was ruled out of the differential diagnosis for the APS following this experiment, as seropositivity was not associated with presence or absence of APS in challenged birds and AE virus could not be detected in brain or spleen tissue of broilers affected with the APS. Recent work (Boros *et al.,* 2014; Lau *et al.,* 2014) has found evidence of a range of genetically distinct picornaviruses (in addition to AE) in clinically normal poultry populations, which is raises the potential of such being implicated in emerging avian diseases. However, several conserved structures exist between these distinct viruses (Boros *et al.,* 2014) and thus it would be expected that such would be detected in the NGS study reported in Chapter 9 if they were indeed present.

Flaviviral disease was included in the differential diagnosis for the APS because WNV (a flavivirus) has been shown to induce CNS pathology in chickens, Kunjin virus (a flavivirus) has induced neurological syndrome in Australian equine populations recently (Chapter 4) and Murray Valley encephalitis virus (a flavivirus native to Australia) in rare cases induces viral encephalitis in humans. Brain and spleen tissue from experimental broiler chickens affected with the APS were tested for the presence of flaviviruses. The PCR test used is regarded as a pan-flavivirus assay in that it is capable of detecting a wide range of viruses in the *Flaviviridae* family due to amplification of part of the *NS5* gene (Kuno *et al.,* 1998) which is highly conserved in flaviviruses. This testing failed to detect the presence of a flavivirus in any sample and thus flaviviral disease was ruled out of the differential diagnosis for the APS. Togaviruses, part of the arboviral family *Togaviridae*, were not considered in the differential diagnosis for the APS,

despite them being known to induce neurological disease in avian species (Guy & Malkinson, 2008). This is because togaviruses are not known to affect animals in Australia and the most common togavirus in Australia affecting humans, Ross River fever virus, does not induce neurological disease in humans.

Auto-immune encephalitis is becoming increasingly better understood in human medicine. There are several forms which result from the presence of antibodies against neuronal proteins involved in synaptic transmission, plasticity or neuronal excitability (Armangue *et al.,* 2012). Examples include anti-NMDA receptor encephalitis and limbic encephalitis, both of which are commonly paraneoplastic syndromes in adults, but not children (Armangue *et al.,* 2012). In veterinary medicine, non-infectious forms of encephalitis are reported predominantly in Pug dogs (Greer *et al.,* 2010), but also in some other dog breeds (Cooper *et al.,* 2014). This encephalitis is described as necrotising meningoencephalitis (NME). Whilst the aetiology of canine NME is unknown, it is thought to have an auto-immune component (Greer *et al.,* 2010). Canine NME is typically characterised by lymphoplasmacytic or histiocytic meningoencephalitis, cerebrocortical necrosis and the absence of any visible infectious agents (Cooper *et al.,* 2014). It is considered unlikely that the APS is a non-infectious encephalitis, given the pattern of disease reproduction achieved in this experiment.

10.4 Further attempts at experimental reproduction of the APS (Chapters 7 and 8)

Further attempts at reproducing the APS were less successful than in 13-C-REP1. The APS was not reproduced at all in two (13-C-REP2 and 13-C-REP3) of the three experiments reported in Chapter 7 or in the experiment reported in Chapter 8 (14-C-REP5). In the third experiment reported in Chapter 7 (13-C-REP4) the APS was only reproduced in one chicken. The experimental reproduction model adopted was based on 13-C-REP1 which showed that the APS could be reproduced in broiler chickens challenged at 21 days of age with spleen cells or whole blood harvested from affected chickens or exposed to litter collected from affected sheds. There are a number of plausible reasons for the low rate of induction of APS, as discussed in Chapters 7 and 8 and these are discussed below.

Figure 10.1 presents mean chicken bodyweights from each of the experiments reported in this thesis along with mean weekly bodyweights for field flocks with and without the APS as reported in Chapter 5. In Figure 10.1 weights between measurement ages are interpolated. It is apparent that the bodyweights achieved in 13-C-REP1 are higher than all other experiments and closest to

field weights. The bodyweights in the three experiments in which the APS was not reproduced are obviously lower than field weights and those from 13-C-REP1. This observation could explain the failure to reproduce the APS in three of the five reported experiments. Epidemiological analyses showed that high mean flock bodyweights at 14, 21 and 28 days of age are significant risk factors for the APS. Thus with the bodyweight profiles shown in Figure 10.1 it is not surprising that the APS failed to be reproduced in three of the five experiments and in only one chicken in 13-C-REP4.

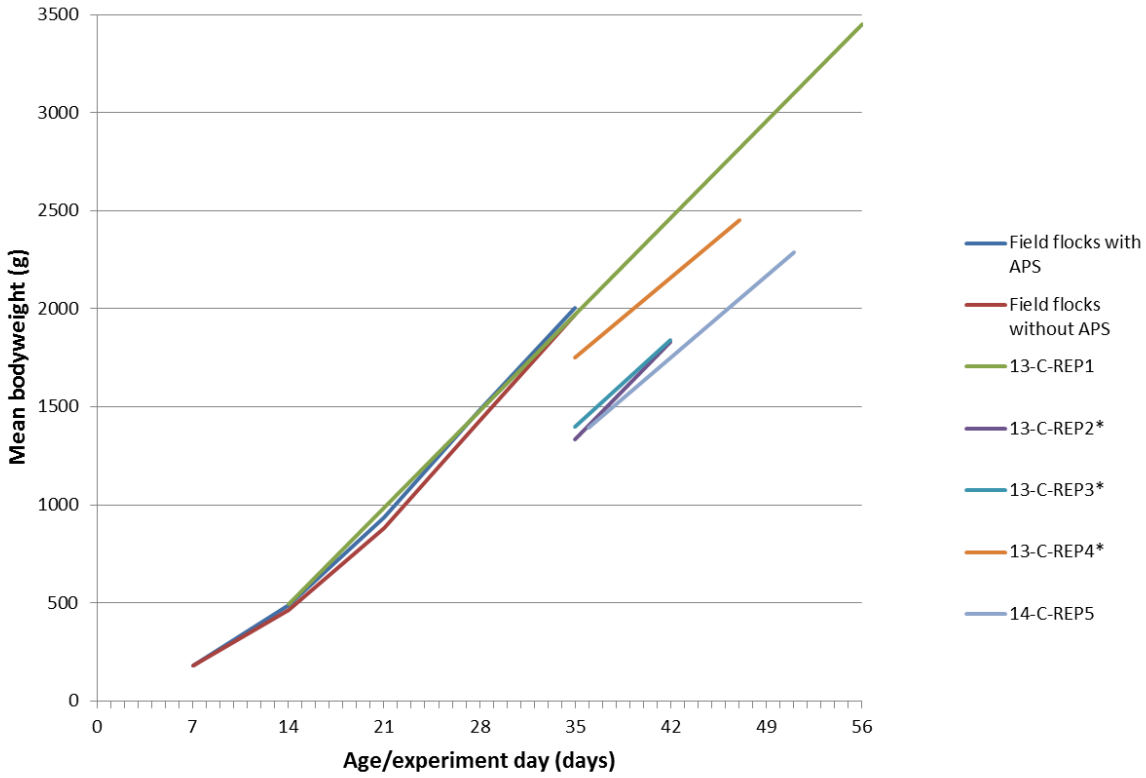

Figure 10.1 Bodyweight comparisons between field and experimental flocks. *Only males were run in these experiments whereas weights for other experimental and field data are mixed sex data.

The three worst performing experiments in terms of bodyweights (Figure 10.1) all had litter trays placed on top of the normal stainless steel isolator floor (Chapter 3), as opposed to the other two experiments which did not. Although done for welfare considerations and to better simulate field conditions the modifications may have contributed to reduced growth rates for two reasons. Firstly, it is likely that air quality was reduced as air flow through each isolator would have been reduced in the presence of a litter tray with the air in-flow being above the floor and the air out-flow being below the floor. Reduced air quality, particularly associated with the presence of elevated levels of ammonia, has been associated with reduced broiler growth rates (Bessei, 2006; Feddes *et al.*, 2003; Zhang *et al.*, 2011). Secondly, the isolator design was such that there was no ability for the feeders to be raised as chickens grew, thus feed outlets often became filled with

litter particularly in the first experiment of this kind. Despite numerous daily efforts to remove litter from feed outlets, it may have been the case that some feed intake restriction occurred in the experiments in which litter trays were used. In later experiments the feeders were raised somewhat more above the level of the litter but litter contamination of the feeders continued, albeit to a reduced extent.

In addition to bodyweight differences between experiments, other possible reasons for the inability to reproduce the APS reliably in each experiment could be differences in challenge materials and source parent flocks used in each experiment. The different batches of challenge material used throughout the experiments may have contained varying amounts of the infective agent/s. However, some level of consistency could have been expected for each as challenge material was only collected from chickens/sheds at the advanced stage of the APS. For this reason, it is considered unlikely that the absence or sub-infective doses of the infective agent(s) within the challenge material resulted in reduced ability of reproducing the APS in experiments performed after 13-C-REP1.

Table 10.1 provides the parent flock identification for the broiler chickens used in each of the experiments reported in this thesis. Variations in parent flocks between experiments could explain the apparent difficulty in reliably reproducing the APS based on variations in protective maternal immunity to the infective agent/s of the APS provided to chickens. It could also be expected that variations in the provision of maternal immunity may vary based on the age of the parent flock, as antibody levels would be expected to fall as the parent flock ages. However, with a diagnosis still not available and restricted epidemiological data on the relationship between particular parent flocks and field cases of the APS, it is not possible to analyse this any further.

Table 10.1 Identification of parent flocks of broiler chickens used in each experiment and extent of APS induced in groups inoculated or exposed to putatively infective materials.

Experiment	**Parent flock**	**Proportion of APS positive chickens in 21 day of age inoculated/exposed chicken groups**
13-C-REP1	B	27 %
13-C-REP2	C	0 %
13-C-REP3	C	0 %
13-C-REP4	A	3 %
14-C-REP5 – Batch 1	D	0 %
14-C-REP5 – Batch 2	B	0 %

A total for 2/3 spinal cords examined from chickens affected with the APS from 13-C-REP1 demonstrated Wallerian degeneration. Similar pathology was observed in 37 % of spinal cord submissions from field chickens affected with the APS (Chapter 4). At this point in the investigation, it was considered that the Wallerian degeneration was potentially associated with

the APS and likely due to trauma caused to neurons associated with inflammation in the CNS. However, as mentioned earlier in this discussion, the neurological examination findings were not consistent with the observed pathology as spinal reflexes were intact. In 13-C-REP4, Wallerian degeneration was observed in the spinal cord of the one chicken which developed the APS and also in two in-contact sham-challenged surviving chickens. This observation was suggestive that the Wallerian degeneration was not associated with the APS. This was not conclusive because sham-challenged chickens were in-contact, thus horizontal transfer of infective agent/s could have occurred, which may have induced a degree of spinal cord pathology. In 14-C-REP5, Wallerian degeneration was observed in the spinal cords of 3/3 blood challenged and clinically normal chickens and 3/3 sham-challenged and clinically normal chickens. The result of this series of findings in relation to the observation of Wallerian degeneration in broiler spinal cords is that the finding is incidental to the presence of the APS. Literature does not exist reporting the presence of Wallerian degeneration in the spinal cords of clinically normal chickens. A likely scenario is that it is normally present to a degree in broiler chickens, due to the rapid remodelling required for rapidly growing chickens.

Given the difficultly in consistently reproducing the APS, chemically induced immunosuppression was attempted as a means of enhancing the ability to reproduce the APS (Chapter 8). With the pattern of reproduction in 13-C-REP1 showing that there is an infectious component to the APS, it was predicted that immunosuppressed chickens would be more susceptible to the APS. Dexamethasone (DEX) and cyclophosphamide (CY) were selected as immunosuppressive agents due to their predominant effects on cell-mediated and humoral immunity respectively (Isobe & Lillehoj, 1993; Lerman & Weidanz, 1970). Effectiveness of the DEX administration was confirmed by serum glucose concentrations in DEX treated chickens on the day of challenge being significantly greater than sham-treated chickens. The DEX treatment did not reduce the survivability of chickens after challenge compared to the sham-treatment chickens.

The CY treatment protocol used resulted in a significant reduction in terminal body and bursal weights compared to all other treatment groups and a reduction in survivability of CY treated chickens compared to all other treatment groups. The CY treatment increased the susceptibility of chickens to CAV and fowl adenovirus serotype 8 (FAV8), which were likely transmitted via the challenge materials and vertically. Despite this, the APS was not reproduced in any chicken in this experiment. The inability to reproduce the APS despite the effective immunosuppression of chickens in this experiment alludes to the complexity of the APS.

Serological testing was performed in the four experiments reported in Chapters 7 and 8 for AE virus, IBD virus, FAV8, Reovirus and CAV given the variable proportions of reactors to each assay reported from 13-C-REP1. Reactors to the AE virus ELISA were not found in any sample tested from the three experiments reported in Chapter 7 (13-C-REP2-4) and only in two samples from 14-C-REP5. Thus interestingly, AEV was more associated with 13-C-REP1.

There was not any evidence of IBD virus ELISA reactivity in broilers in any experiment reported in this thesis. However, SPF chickens demonstrated varying levels of reaction in 13-C-REP1, showing the presence of IBD virus in challenge materials. Maternal antibody (MAb) levels likely prevented seroconversion in broilers. Whilst MAb levels typically decay to below protective levels from 14 days of age as earlier discussed, Table 7.12 showed that MAb levels were still high at 14 days of age in the chickens used in 13-C-REP4. This is likely a result of the IBD virus vaccination regimes used in parent flocks supplying the region.

Serum samples collected from all experiments were tested for FAV8 and the number of samples reacting varied. None of the samples collected from 13-C-REP4 reacted in this assay. There were no obvious differences in FAV8 serology between the experiments in which the APS was reproduced and those in which it was not. Seroconversion to Reovirus was detected in small numbers of chickens in all experiments except for 13-C-REP2 and 13-C-REP3. Seroconversion to CAV was observed in chickens from all experiments and the virus was transmitted vertically and via the challenge materials. There was no significant variation in CAV serology between the experiments in which the APS was reproduced and those in which it was not.

10.5 Advanced molecular investigations into the APS (Chapter 9)

Whole genome next generation sequencing (NGS) was pursued in this case because of the possibility of the APS being caused by an infectious agent at which earlier diagnostic investigations had not targeted. Spleen tissue was the predominant source tissue from which DNA and RNA were extracted for NGS. Spleen tissue was selected because it was an effective mode of transmission for the APS as reported in Chapter 6. Additionally, the quality and yield of DNA and RNA extractions from spleen tissue were typically much higher than from blood. The sequencing yield from all samples was good except from one of the RNA samples. The accuracy of the sequencing reads from the submitted samples was high based on the Phred scoring system, for each sample the accuracy of base calls was at least 99.9 %.

Two levels of screening were employed in order to detect and match nucleic acid (NA) sequences in the sequenced samples. The Kraken software was the only level employed in order to detect NA sequences of bacterial origin and was also used as an initial approach to detecting NA sequences of viral origin. The Kraken approach is regarded as having high classification accuracy and precision (Wood & Salzburg, 2014). Based on the greater similarity between numerous known viral diseases of poultry and the APS (Chapter 2, 4), an additional level of screening was employed to detect NA sequences of viral origin in order to increase the sensitivity of bioinformatics. This involved constructing a basic local alignment search tool (BLAST) library from assembled un-aligned reads and searching such for the presence of NA sequences specific to viral protein sequences recorded in RefSeq (National Centre for Biotechnology Information, USA).

From the Kraken search, the presence of bacteria was found in a number of samples. The presence of all bacteria detected could be explained by contamination during post-mortem sampling except that of *Dichelobacter spp.* in one sample. This was believed to be most likely a laboratory contaminant. The Kraken search additionally detected feline herpesvirus-1 (FHV1) in one affected chicken sample.

From the BLAST search, for each sample, only the matches of highest sequence similarity were considered. Then, all sequence matches from control chicken samples and corresponding matches from affected chicken samples were excluded. The remaining viral protein matches were then visually assessed. From the DNA samples, sequences specific to FHV1 were detected in the same sample in which FHV1 was detected from the kraken search and also in two additional samples. Based on this finding, performing a FHV1 specific PCR (Persico *et al.,* 2011; Sandmeyer *et al.*, 2010) could follow in order to screen a large number of chicken samples for the presence of FHV1. From the RNA samples, numerous viral protein sequences were detected for CAV, invertebrate iridescent virus (IIV) and respiratory syncytial virus (RSV). Given that evidence of CAV, IIV and RSV was not detected in the Kraken search it is likely that the presence of each was at low levels. CAV was known to be in the challenge material and 30 % of chickens affected with the APS were seropositive to CAV in the experiment 13-C-REP1. IIV's are not known to induce disease in avian species. The presence of viral protein matches to IIV in several affected chicken samples is interesting; however, a consistent viral protein match was not observed in each positive chicken sample which would make the design of a PCR test to further screen samples difficult. Viral protein matches to RSV with high similarity were detected in three of five affected chicken spleen samples. The development of a reverse transcriptase (RT)-PCR based on the RSV non-structural protein 1 (1C) which was found in each positive sample and 100 % identical to the

recorded sequence, represents a logical next step in order to screen a large number of chicken tissue samples for the presence of RSV.

10.6 Summary of findings

1. Chickens affected with the APS clinically demonstrated sternal recumbency, stupor and marked flaccidity of the neck at the most advanced stage of the syndrome.
2. Mortality and cull rates were significantly higher in affected flocks.
3. Histopathological changes were observed in the cerebrum of affected chickens, characterised by non-suppurative vasculocentric encephalitis.
4. Epidemiological investigations indicated that the most important risk factors associated with the presence of the APS included:
    a. Below standard brooding temperatures in the first seven days of age;
    b. Larger flock sizes;
    c. High external temperatures through the life of a flock; and
    d. Greater than average increases in maximum shed temperatures as outside temperatures increase.
5. Additional identified risk factors of relevance associated with the presence of the APS included:
    a. Flock bodyweight increases above the Ross 308 breed standards;
    b. High maximum and fluctuations in daily shed temperatures;
    c. High fluctuations in external temperatures; and
    d. Delays in flock age at 1st thin-out.
6. Males were more susceptible to the APS than were females;
7. Ross 308 chickens may have a genetic predisposition to the APS as APS-like syndromes have been reported anecdotally elsewhere in this strain and the APS could not be reproduced in SPF chickens.
8. The APS is caused at least in part by the presence of an infectious agent(s) because:
    a. It could be reproduced experimentally in Ross broiler chickens by inoculating 21 day chickens with spleen cells or blood harvested from affected chickens, or exposing 21 day old chickens to litter from affected sheds;
    b. The susceptibility of 21 day of age chickens and not one-day old chickens to the APS is consistent with decline of maternal antibody; and
    c. The observed brain histopathology was most similar to other viral infections of poultry including MDV, AE virus, ND virus, AI virus and flaviviruses, each of which have been ruled out as causative agents.

9. Several findings were inconsistent with the APS being of infectious aetiology, including:
    a. The APS could not be reliably experimentally reproduced, and
    b. Viral particles were not observed associated with brain lesions from electron microscopy and bacteria were not observed in samples of any kind under light microscopy.
10. Results of interest from NGS were the evidence of FHV1 and RSV in a proportion of affected chicken samples which were not observed in un-affected sham-challenged chicken samples. The RSV non-structural protein 1 (1C) was present in all positive samples and 100 % identical to the recorded sequences for that protein in each sample.

10.7 Future work

This section will briefly explore two concepts which were largely outside the scope of this thesis but which may be relevant to further investigations before summarising the key points relevant to further investigations into the APS.

10.8.1 Early litter exposure, age associated resistance and the polio model

The concept of the *polio model* may be relevant to attempting to understand the APS. Polio (poliomyelitis or infantile paralysis) is a disease caused by poliovirus which is an enterovirus that is capable in invading local lymphoid tissue and entering the blood stream (Bunimovich-Mendrazitsky & Stone, 2005). Exposure of humans to poliovirus results in the absence of symptoms in 72 % of cases, minor illness in 24 % of cases, non-paralytic poliomyelitis in 4 % of cases and paralytic poliomyelitis in less than 1 % of cases (Sutter *et al.,* 1999). Throughout the history of polio the occurrence of infection shifted from endemic to epidemics as the human population developed. In the pre-epidemic era it is thought that there was more consistent exposure to the virus and thus most infections occurred in infancy and the disease remained endemic (Nielsen *et al.,* 2002). The model proposes that as civilisation developed, personal hygiene and population sanitation improved and family sizes became smaller and thus it is thought that exposure to the virus often did not occur in infancy leaving teenagers and adults susceptible to the virus and thus the population was susceptible to epidemics of the disease (Nielsen *et al.,* 2002). It has been reported that the proportion of paralytic cases in the exposed population increases with the age of the population (Nathanson & Martin, 1979; Miller & Gay, 1997). Hence the model proposes that greater incidence of polio is associated to delayed and inconsistent exposure to the virus.

In relation to the APS, broiler farm biosecurity and sanitation practices have improved markedly in recent history. Broiler farms in the region affected by the APS are sanitised thoroughly and litter replaced between chicken batches and thus it is conceivable that exposure to an infectious agent could be inconsistent, particularly in new design sheds with concrete flooring. Additionally compared to past times, exposure to a putative infectious agent would likely occur later in the life of a broiler flock if infection was to occur.

Of further relevance to this discussion are two additional enteroviruses of humans, A71 and D68 which have both been associated with acute flaccid paralysis in humans (Huang *et al.*, 1999; Mirand & Peigue-Lafeville, 2015). If an enterovirus was associated with the APS it could have been detected via the NGS reported in Chapter 9. However, given that its sensitivity was only 63%, it is possible that NGS failed to detect such.

Results from 13-C-REP1 demonstrated that chickens which were challenged with the putative agent/s at/from hatch did not develop the APS whereas those challenged at/from 21 days of age did develop the APS. As discussed in Chapter 6 this apparent early protection may be a result of protective MAb. The relevance of the concept of the *polio model* in relation to the APS is if there was any early age resistance to the APS in addition to the likely MAb effects already discussed. This concept could have been further tested in-part from the design of the experiment 13-C-REP2 by exposing chickens to contaminated litter at placement and subsequently challenging them at 21 days of age; however, unfortunately the APS could not be reproduced in that experiment. The relevance of the *polio model* to the APS is that litter re-use may be beneficial in reducing the incidence of the APS. Similarly early exposure in the presence of MAb and the subsequent development of adaptive immunity could provide protection. This idea provides further grounds for investigation and on-farm experiments over time represent a more feasible platform for such experiments given the difficulty in reliably reproducing the APS in isolator facilities.

10.8.2 Traditional viral isolation techniques

Attempts at virus culture from potentially infective tissues associated with the APS were not in the scope of this thesis. However, future work in relation to the APS may consider these techniques. Given host specificity of many viruses, attempts at virus culture in relation to the APS would ideally be performed in embryonated SPF chicken eggs or derived cells such as chicken embryo fibroblasts (CEF). Inoculated chicken embryos would then be observed for changes associated with viral infection such as mortality, deformity or haemorrhage associated with the embryo or lesions associated with the embryo membranes. Similarly, inoculated cells lines would

be observed for abnormalities that would be consistent with viral infection. In the event of propagation, further diagnostics such as electron microscopy and NGS could be utilised.

In relation to the APS, Wajid (2013) attempted viral culture by inoculating CEF's with cryopreserved spleen cells harvested from affected chickens from farm 14 prior to commencement of work in this thesis. He reported that no typical MDV1 plaques were visible within the CEF cultures after three passages; however, no further comment was provided in relation to other possible changes such as cytopathic effects or heamadsorption ability.

The issue with the culture approach is that the material that would be used to inoculate embryos or cell lines would be spleen cells or blood harvested from affected chickens. It would be likely that such material would contain a range of viruses, including IBD virus, FAV8 and CAV (shown by SPF chickens seroconverting to each subsequent to challenge in 13-C-REP1) in addition to the putative infectious agent(s) associated with the APS. This issue obviously complicates the interpretation of any changes which may be observed in inoculated embryos or cell lines which may indicate viral propagation.

10.8.3 Summary of future work

1. Further attempts at experimental reproduction need to be conducted with a focus on achieving chicken bodyweights at or above the Ross 308 breed standards in order to maximise the chances of disease reproduction.
2. Epidemiological investigations should continue over a longer time period and should include data collected relevant to Ross broiler chickens produced in other production regions. This will increase the relevance of findings particularly if proposed risk factors from the affected region are compared to figures relating to unaffected production regions.
3. Modifications may be considered at the farm level based on the identified risk factors from epidemiological investigations, including:
    a. Continuous monitoring of brooding temperatures in the first week of life such that deviations from the standard guidelines can be corrected quickly,
    b. Tighter control of shed temperatures through the life of the flock to minimise deviations above maximum shed temperatures as per the Ross 308 breed standard guidelines and to minimise daily fluctuations in shed temperature,
    c. Closer regulation of thin out age in order to avoid the first thin out occurring after 35 days of age, and
    d. Growth restriction through the life of the flock in order to minimise deviations above the Ross 308 breed standards.

4. Based on NGS findings PCR tests could be performed to screen larger numbers of chicken samples for further investigation. Numerous published assays for FHV1 are available which could be adopted for use in this case. Based on the presence of RSV non-structural protein 1 (1C) in all positive samples being 100 % identical to the recorded sequences for that protein, an RT-PCR should be designed based on this.
5. Investigations relating to the possibility of the APS being a form of enterotoxaemia (for example by screening chicken samples utilising a *C. perfringens* type D toxin ELISA) or caused by an enterovirus.
6. On-farm experiments could be conducted to investigate protective effects afforded by early exposure of a flock to contaminated shed litter. This would ensure exposure to the putative agent when susceptibility is low due to possible maternal antibody and/or age effects.
7. Attempts at viral culture further to that reported by Wajid (2013) may be considered.

References

ACMF (2013). Industry facts & figures. Australian Chicken Meat Federation Inc, North Sydney. Retrieved 9th February, 2015, http://www.chicken.org.au.

Alexander, D. J., & Senne, D. A. (2008). Newcastle Disease. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 75-100). Ames, Iowa: Blackwell.

Almquist, H. J. (1942). Magnesium requirement of the chick. *Experimental Biology and Medicine,* 49, 544-545.

Alsharari, M., Islam, A.F.M.F., Walkden-Brown, S.W. & Renz, K.G. (2015). Tissue distribution and shedding profiles of chicken anaemia virus in specific pathogen-free and commercial broiler chickens. *Proceedings of the Australian Poultry Science Symposium* (pp. 179-182), Sydney: University of Sydney.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* 515(3), 403-410.

Anderson, D.E., Lush, J.L. & Chambers, C. (1957). Studies on bovine ocular squamous carcinoma (‘cancer eye’). II. Relationships between eyelid pigmentation and occurrence of cancer eye lesions. *Journal of Animal Science,* 16, 739-746.

Armangue, T., Petit-Pedrol, M. & Dalmau, J. (2012). Autoimmune encephalitis in children. *Journal of Child Neurology,* 27(11), 1460-1469.

Aviagen (2012). Ross 308 broiler performance objectives. Huntsville, USA.

Barnes, H. J., Nolan, L. K., & Vaillancourt, J.-P. (2008). Colibacillosis. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 691-732.). Ames, Iowa: Blackwell Publishing.

Bartov, I. (1989). Lack of effect of dietary factors in nicarbazin toxicity in broiler chicks. *Poultry Science,* 68(1), 145-152.

Barzon, L., Lavezzo, E., Costanzi., G., Franchin, E., Toppo, S. & Palu, G. (2013). Next-generation sequencing technologies in diagnostic virology. *Journal of Clinical Virology,* 58, 346-350.

Bessei, W. (2006). Welfare of broilers: a review. *Worlds Poultry Science Journal,* 62(3), 455-466.

Biancifiori, F., Rondini, C., Grelloni, V., & Frescura, T. (1986). Avian toxoplasmosis: experimental infection of chicken and pigeon. *Comparative immunology, microbiology and infectious diseases*, 9(4), 337-346.

Bickford, A. A., & Saunders, J. R. (1966). Experimental toxoplasmosis in chickens. *American Journal of Veterinary Research*, 27(116), 308-318.

Blood, D.C., Henderson, J.A., Radostits, O.M., Arundel, J.H. & Gay, C.C. (1979). *Veterinary Medicine Fifth Edition* (pp. 1087-1089). London, Bailliere Tindall.

Bolton, T. B. (1971). The structure of the nervous system In D. J. Bell & G. M. Freeman (Eds.), *Physiology and biochemistry of the domestic fowl* (pp. 641-673). London: Academic Press.

Boros, A., Pankoviks, P. & Reuter, G. (2014). Avian picornaviruses: molecular evolution, genome diversity and unusual genome features of a rapidly expanding group of viruses in birds. *Infection, Genetics & Evolution,* 28, 151-166.

Bowes, V. A., Julian, R. J. & Stirtzinger, T. (1989). Comparison of serum biochemical profiles of male broilers with female broilers and white Leghorn chickens. *Canadian Journal of Veterinary Research,* 53, 7-11.

Bragg, D. B., Salem, H. A., & Devlin, T. J. (1970). Effect of dietary triticale ergot on the performance and survival of broiler chicks. *Canadian Journal of Animal Science,* 50, 259-264.

Bumstead, N. & Barrow, P.A. (1988). Genetics of resistance to Salmonella typhimurium in newly hatched chicks. *British Poultry Science,* 29(3), 521-529.'

Bumstead, N., Reece, R.L. & Cook, J.K. (1993). Genetic differences in susceptibility to chicken lines to infection with infectious bursal disease virus. *Poultry Science,* 72(3), 403-410.

Bunimovich-Mendrazitsky, S. & Stone, L. (2005). Modelling polio as a disease of development. *Journal of Theoretical Biology,* 237, 302-315.

Calnek, B. W. (2008). Avian Encephalomyelitis. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 430-441). Ames, Iowa: Blackwell.

Cardona, C.J., Bickford, A.A., Charlton, B.R. & Cooper, G.L. (1993). Enterococcus durans infection in young chickens associated with bacteraemia and encephalomalacia. *Avian Diseases*, 37, 234-239.

Caskey, C. D., Norris, L. C., & Heuser, G. F. (1944). A chronic congenital ataxia in chicks due to manganese deficiency in the maternal diet. *Poultry Science*, 23, 516-520.

Chandriani, S., Skewes-Cox, P., Zhong, W., Ganem, D.E., Divers, T.J., Van Blaricum, A.J., Tennant, B.C. and Kistler, A.L. (2013). Identification of a previously undescribed divergent virus from the Flaviviridae family in an outbreak of equine serum hepatitis. *Proceedings of the National Academy of Sciences USA*, 110(15), E1407-1415.

Charlton, B. R., Chin, R. P., & Barnes, H. J. (2008). Fungal Infections. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 989-1008). Ames, Iowa: Blackwell Publishing.

Chen, K.L, Tsay, S.M., Chiou, P.W., Sun, C.P. & Weng, B.C. (2010). Effects of caponization and different forms of exogenous androgen implantation on immunity in male chicks. *Poultry Science,* 89(5), 887-894.

Chu, H.P. & Rizk, J. (1975). The effect of maternal immunity, age at vaccination and doses of live vaccines on immune response to Newcastle disease. *Developments in Biological Standardization.* 28, 451-463.

Clark, W. E. (1987). Avian botulism. In M. W. Eklund & V. R. J. Dowell (Eds.), *Avian Botulism: An International Perspective*. (pp. 89-105). Springfield, IL: Charles C. Thomas.

Clippinger, T. L., Bennett, A., & Platt, S. R. (1996). The avian neurologic examination and ancillary neurodiagnostic techniques. *Journal of Avian Medicine and Surgery,* 10(4), 221-247.

Cooper, G. L. (1989). An encephalitic form of listeriosis in broiler chickens. *Avian Diseases*, 33(1), 182-185.

Cooper, J.J., Schatzberg, S.J., Vernau, K.M., Summers, B.A., Porter, B.F., Siso, S., Young, B.D. & Levine, J.M. (2014). Necrotizing meningoencephalitis in atypical dog breeds: a case series and literature review. *Journal of Veterinary Internal Medicine.* 28(1), 198-203.

Corboy, J.M. & Tyler, K.L. (2004). Neurovirology. In W.G. Bradley, R.G. Daroff, G.M. Fenichel & J. Jankovic (Eds.), *Neurology in Clinical Practice: Principles of Diagnosis and Management 4th Edition* (pp. 831-848). Philadelphia: Butterworth Heinemann.

Crespo, R., & Shivaprasad, H. L. (2008). Developmental, Metabolic, and Other Noninfectious Disorders. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 1149-1195). Ames, Iowa: Blackwell Publishing.

Davis, J. F. (2008). Hypoglycaemia-Spiking Mortality Syndrome of Broiler Chickens. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 1269-1272). Ames, Iowa: Blackwell Publishing.

De Lahunta, A. (1983). *Veterinary neuroanatomy and clinical neurology 2nd edition* (pp. 77-242). Philadelphia: WB Saunders.

Dohms, J.E. (1987). Laboratory investigation of botulism in poultry. In M.W. Eklund & V.R. Dowell (Eds.), *Avian botulism: an international perspective* (pp. 295-314) Springfield, Illinois: Charles C. Thomas.

Dohms, J.E. (2008). Botulism. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan & D.E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 879-885). Ames, Iowa: Blackwell Publishing.

Dunowska, M., Biggs, P.J., Zheng, T. & Perrott, M.R. (2012). Identification of a novel nidovirus associated with a neurological disease of the Australian brushtail possum (*Trichosurus vulpecula*). *Veterinary Microbiology*, 156(3-4), 418-424.

Eidson, C.S., Ellis, M.H. & Kleven, S.H. (1981). Reduced vaccinal protection of turkey herpesvirus against field strains of Marek's disease herpesvirus. *Poultry Science,* 60(2), 317-322.

Evans, A. S. (1976). Causation and disease: the Henle-Koch postulates revisited. *The Yale Journal of Biology and Medicine*, 49(2), 175-195.

Ewing, B. & Green, P. (1998). Base-calling of automated sequencer traces using Phred. II. Error probabilities. *Genome Research,* 8, 186-194.

Fadly, A. M., & Nair, V. K. (2008). Leukosis/Sarcoma Group. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 514-568). Ames, Iowa: Blackwell Publishing.

Feddes, J.J.R., Emmanuel, E.J., Zuidhof, M.J. & Korver, D.R. (2003). Ventilation rate, air circulation, and bird disturbance: effects on the incidence of cellulitis and broiler performance. *Journal of Applied Poultry Research,* 12(3), 328-334.

Fenner, W. R. (1995). Diseases of the brain. In S. J. Ettinger & E. C. Feldman (Eds.), *Veterinary Internal Medicine 4th Edition* (pp. 578-629). Philadelphia: WB Saunders.

Fitzmaurice, G. (2003). Confused or confounding? *Nutrition,* 19(2), 189-191.

Flanders, W.D., Klein, M. & Mirabelli, M.C. (2016). Conditions for valid estimation of causal effects on prevalence in cross-sectional and other studies. *Annals of Epidemiology,* 26, 389-394.

Frost, M.J., Zhang, J., Edmonds, J.H., Prow, N.A., Gu, X., Davis, R., Hornitzky, C., Arzey, K.E., Finlaison, D., Hick, P., Read, A., Hobson-Peters, J., May, F.J., Doggett, S.L., Haniotis, J., Russell, R.C., Hall, R.A., Khromykh, A.A. & Kirkland, P.D. (2012). Characterisation of Virulent West Nile Virus Kunjin Strain, Australia, 2011. *Emerging Infectious Diseases,* 18(5), 792-800.

Fulton, R. M. (2008). Other Toxins and Poisons. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 1231-1258.). Ames, Iowa: Blackwell Publishing.

Funk, K.A., Henderson, J.D., Liu, C.H., Higgins, R.J. & Wilson, B.W. (1994). Neuropathology of organophosphate-induced delayed neuropathy (OPIDN) in young chickens. *Archives of Toxicology,* 68(5), 308-316.

Gharaibeh, S. & Mahmoud, K. (2013). Decay of maternal antibodies in broiler chickens. *Poultry Science.* 92(9), 2333-2336.

Gimeno, I. M., Witter, R. L., Hunt, H. D., Lee, L. F., Reddy, S. M., & Neumann, U. (2001). Marek's disease virus infection in the brain: Virus replication, cellular infiltration, and major histocompatibility complex antigen expression. *Veterinary Pathology*, 38(5), 491-503.

Glisson, J. R., Hofacre, C. L., & Christensen, J. P. (2008). Fowl Cholera. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 739-758). Ames, Iowa: Blackwell Publishing.

Goodwin, M. A., Bounous, D. I., Brown, J., McMurray, B. L., Ricken, W. L., & Magee, D. L. (1994). Blood glucose values and definitions for hypoglycaemia and hyperglycemia in clinically normal broiler chicks. *Avian Diseases*, 38(4), 861-865.

Gough, R. E., & Jones, R.C. (2008). Avian metapneumovirus. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 100-106). Ames, Iowa: Blackwell.

Guy, J.S. & Malkinson, M. (2008). Arbovirus Infections. In Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan & D.E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 414-425). Ames, Iowa: Blackwell Publishing.

Greer, K.A., Wong, A.K., Liu, H., Famula, T.R., Pedersen, N.C., Ruhe, A., Wallace, M. & Neff, M.W. (2010). Necrotizing meningoencephalitis of Pug dogs associates with dog leukocyte antigen class II and resembles acute variant forms of multiple sclerosis. *Tissue Allergens,* 76(2), 110-118.

Gries, C. L., & Scott, M. L. (1972). The pathology of pyridoxine deficiency in chicks. *Journal of Nutrition,* 102(10), 1259-1267.

Hafez, Y.S.M., Chavez, E., Vohra, P. & Kratzer, F.H. (1978). Methionine toxicity in chicks and poults. *Poultry Science,* 57, 699-703.

Hall, A.G. & Tilby, M.J. (1992). Mechanisms of action of, and modes of resistance to, alkylating agents used in the treatment of haematological malignancies. *Blood Reviews,* 6(3), 163-173.

Hasman, H., Saputra, D., Sicheritz-Ponten, T., Lund, O., Svendsen, C.A., Fromodt-Moller, N. & Aarestrup, F.M. (2014). Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. *Journal of Clinical Microbiology,* 52(1), 139-146.

Hernan, M.A. (2004). A definition of causal effect for epidemiological research. *Journal of Epidemiology & Community Health,* 58, 265-271.

Hess, W.R. (1981). Comparative aspects and diagnosis of the iridoviruses of vertebrate animals. In Kurstak, E. & C. Kurstak (Eds.), *Comparative diagnostics of viral diseases volume III – Vertebrate animal and relative viruses.* New York: Academic Press.

Hoffmann, B., Scheuch, M., Hoper, D., Jungblut, R., Holsteg, M., Schirrmeier, H., Eschmaumer, M., Goller, K.V., Wernike, K., Fischer, M., Breithaupt, A., Mettenleiter, T.C. & Beer, M. (2012). Novel Orthobunyavirus in Cattle, Europe, 2011. *Emerging Infectious Diseases,* 18(3), 469-472.

Hosmer, D.W. & Lemeshow, S. (2000). *Applied Logistic Regression Second Edition* (pp. 1-143). New York: John Wiley & Sons Inc.

Huang, C., Liu, C., Chang, Y., Chen, C., Wang, S & Yeh, T. (1999). Neurological complications in children with enterovirus 71 infection. *New England Journal of Medicine,* 341(13), 936-942.

Huebner, R. J. (1957). Criteria for etiologic association of prevalent viruses with prevalent diseases; the virologist's dilemma. *Annals of the New York Academy of Sciences,* 67(8), 430-438.

Huff, G. R., Huff, W. E., Balog, J. M. & Rath, N. C. (1998). The effects of dexamethasone immunosuppression on turkey osteomyelitis complex in an experimental *Escherichia coli* respiratory infection. *Poultry Science,* 77(5), 654-661.

Hunter, B., & Wobeser, G. (1980). Encephalopathy and peripheral neuropathy in lead-poisoned mallard ducks. *Avian Diseases,* 24(1), 169-178. doi: 10.2307/1589776.

Illumina Inc. 2014. *An introduction to next-generation sequencing technology*. San Diego: Illumina Inc. Accessed 29th April 2015. Available from: http://www.illumina.com/content/dam/illumina-marketing/documents/products/illumina_sequencing_introduction.pdf.

Islam, A., Harrison, B., Cheetham, B. F., Mahony, T. J., Young, P. L. & Walkden-Brown, S. W. (2004). Differential amplification and quantitation of Marek's disease viruses using real-time polymerase chain reaction. *Journal of Virological Methods*, 119(2), 103-113.

Isobe, T. & Lillehoj, H. S. (1993). Dexamethasone suppresses T-cell-mediated immunity and enhances disease susceptibility to *Eimeria mivati* infection. *Veterinary Immunology and Immunopathology*, 39(4), 431-446.

Johnson, R. T., & Gibbs, C. J. (1974). Editorial: Koch's postulates and slow infections of the nervous system. *Archives of neurology*, 30(1), 36-38.

Joiner, K.S., Ewald, S.J., Hoerr, F.J., van Santen, V.L. & Toro, H. (2005). Oral infection with chicken anaemia virus in 4-Wk broiler breeders: lack of effect of major histocompatibility B complex genotype. *Avian Diseases*, 49(4), 482-487.

Jones, R. C. (2008). Viral Arthritis. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 310-322). Ames, Iowa: Blackwell Publishing.

Jordan, F. T., Howell, J. M., Howorth, J., & Rayton, J. K. (1976). Clinical and pathological observations on field and experimental zoalene poisoning in broiler chicks and the effect of the drug on laying hens. *Avian Pathology* 5(3), 175-186.

Kenzy, S.G., Cho, B.R. & Kim, Y. (1973). Oncogenic Marek's disease herpesvirus in avian encephalitis (temporary paralysis). *Journal of the National Cancer Institute*, 51, 977-982.

Keyburn, A.L., Boyce, J.D., Vaz, P., Bannam, T.L., Ford, M.E., Parker, D., Di Rubbo, A., Rood, J.I. & Moore, R.J. (2008). NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens. PLoS Pathogens*, 4(2), E26.

Klasing, K. C. (2008). Nutritional Diseases. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 1121-1148). Ames, Iowa: Blackwell Publishing.

Klein, S.L. (2000). The effects on sex differences in infection: from genes to behaviour. *Neuroscience & Behavioural Reviews*, 24(6), 627-638.

Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L. & Burke, D. S. (1989). Antibody-dependant enhancement of Dengue virus growth in human-monocytes as a risk factor for Gengue Hemorrhagic Fever. *American Journal of Tropical Medicine and Hygiene*, 40(4), 444-451.

Kuno, G., Chang, G. J. J., Tsuchiya, K. R., Karabatsos, N. & Cropp, C. B. (1998). Phylogeny of the genus Flavivirus. *Journal of Virology*, 72(1), 73-83.

Lanzas, C. & Chen, S. (2015). Complex system modelling for veterinary epidemiology. *Preventive Veterinary Medicine.* 118 (2-3), 207-214.

Lau, S.K., Woo, P.C., Yip, C.C., Li, K.S., Fan, R.Y., Bai, R., Huang, Y., Chan, K.H. & Yuen, K.Y. (2014). Chickens host diverse picornaviruses originated from potential interspecies transmission with recombination. *Journal of General Virology,* 95, 1929-1944.

Leach, R. M. J., & Nesheim, M. C. (1963). Studies on chlorine deficiency in chicks. *Journal of Nutrition,* 81, 193-199.

Lecocq, F. R., Mebane, D. & Madison, L. L. (1964). The acute effect of hydrocortisone on hepatic glucose output and peripheral glucose utilisation. *Journal of Clinical Investigation*, 43, 237-246.

Lecuit, M. & Eliot, M. (2014). The diagnosis of infectious disease by whole genome next generation sequencing: a new era is opening. *Frontiers in Cellular and Infection Microbiology*, 4(25), 1-3.

Lerman, S. P. & Weidanz, W. P. (1970). Effect of cyclophosphamide on ontogeny of humoral immune response in chickens. *Journal of Immunology*, 105, 614-619.

Leung, D.Y. & Bloom, J.W. (2003). Update on glucocorticoid action and resistance. *Journal of Allergy and Clinical Immunology*, 111(1), quiz 23.

Li, H & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics,* 25, 1754-1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Hiomer, N., Marth, G., Abecasis, G., Durbin, R. & 1000 Genome Project Data Processing Subgroup (2009). The sequencing alignment/map (SAM) format and SAMtools. *Bioinformatics,* 25, 2078-2079.

Lower Jr, G. M. (1983). Systematic epidemiologic theory: Conceptual foundations and axiomatic elements. *Medical Hypotheses*, 11(2), 195-215.

Macchi, B. d. M., Quaresma, J. A. S., Herculano, A. M., Crespo-Lopez, M. E., DaMatta, R. A., & do Nascimento, J. L. M. (2010). Pathogenic action of Plasmodium gallinaceum in chickens: brain histology and nitric oxide production by blood monocyte-derived macrophages. *Veterinary Parasitology*, 172(1-2), 16-22.

Mackintosh, C.G., Schollum, L.M., Harris, R.E., Blackmore, D.K., Willis, A.F., Cook, N.R. & Stoke, J.C.J. (1980). Epidemiology of leptospirosis in dairy farm workers in Manawatu. Part 1: A cross-sectional serological survey and associated occupational factors. *New Zealand Veterinary Journal,* 28, 245-250.

Maes, R. (2012). Felid herpesvirus type 1 infection in cats: a natural host model for alphaherpesvirus pathogenesis. *International Scholarly Research Network Veterinary Science*, vol. 2012, pp. 1-10.

Mann, C.J. (2003). Observational research methods. Research design II: cohort, cross sectional, and case-control studies. *Emergency Medicine Journal,* 20, 54-60.

Martin, L. T., Martin, M. P., & Barnes, H. J. (2011). Experimental Reproduction of Enterococcal Spondylitis in Male Broiler Breeder Chickens. *Avian Diseases*, 55(2), 273-278.

Mateu, E. & Diaz, I. (2008). The challenge of PRRS immunology. [Review]. *Veterinary Journal*, 177(3), 345-351.

McCrea, C.T. & Head, K.W. (1978). Sheep tumours in north-east Yorkshire. I. Prevalence on seven moorland farms. *British Veterinary Journal,* 134, 454-461.

McKay, L. I. & Cidlowski, J. A. (2003). Pharmacologic effects of corticosteroids. In D. W. Kufe, R. E. Pollock, R. R. Weichselbaum, R. C. Bast, T. S. Gansler, J. F. Holland & E. Frei (Eds.), *Holland-Frei Cancer Medicine 6th Edition.* Hamilton, Ontario: BC Decker.

McNamee, R. (2005). Regression modelling and other methods to control confounding. *Occupational and Environmental Medicine*, 62, 500-506.

Michelsen, P. G. & Smith, B. P. (2009). Diseases caused by Clostridium perfringens toxins. In B. P. Smith (Ed.), *Large Animal Internal Medicine Fourth Edition* (pp. 873-874). St. Louis: Mosby Elsevier.

Miller, E. & Gay, N. (1997). Effect of age on outcome and epidemiology of infectious diseases. *Biologicals,* 25, 137-142.

Mirand, A. & Peigue-Lafeville, H. (2015). Acute flaccid myelitis and enteroviruses: an ongoing story. *The Lancet,* 385, 1601-1602.

Mutalib, A., Riddell, C., & Osborne, A. D. (1983). Studies on the pathogenesis of staphylococcal osteomyelitis in chickens. I. Effect of stress on experimentally induced osteomyelitis. *Avian Diseases*, 27(1), 141-156.

Myers, R. K., & McGavin, M. D. (2007). Cellular and Tissue Responses to Injury. In M. D. McGavin & J. F. Zachary (Eds.), *Pathologic Basis of Veterinary Disease Fourth Edition* (pp. 1-62). St. Louis, Missouri: Mosby Elsevier.

Nakamura, K., Ohtsu, N., Nakamura, T., Yamamoto, Y., Yamada, M., Mase, M. & Imai, K. (2008). Pathologic and immunohistochemical studies of Newcastle Disease (ND) in broiler chickens vaccinated with ND: severe non-purulent encephalitis and necrotising pancreatitis. *Veterinary Pathology*, 45, 928-933.

National Climate Centre (2014). Climate data online. Retrieved 27th October, 2014, from http://www.bom.gov.au/climate/data.

Nathanson, N. & Martin, J.R. (1979). Epidemiology of poliomyelitis – enigmas surrounding its appearance, epidemicity and disappearance. *American Journal of Epidemiology*, 110(6), 672-692.

NCBI (2015). *Taxonomy*. Bethedsa USA: National Centre for Biotechnology Information. Retrieved 31st July 2015, from http://www.ncbi.nlm.nih.gov/taxonomy.

Nielsen, N.M., Aaby, P., Wohlfahrt, J., Molbak, K. & Melbye, M. (2002). The Polio Model. Does it apply to polio? *International Journal of Epidemiology*, 31, 181-186.

Opengart, K. (2008). Necrotic Enteritis. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), Diseases of Poultry 12th Edition (pp. 872-879). Iowa: Blackwell Publishing.

Otaki, Y., Saito, K., Tajima, M. & Nomura, Y. (1992). Persistence of maternal antibody to chicken anemia agent and its effect on the susceptibility of young chickens. *Avian Pathology.* 21(1), 147-151.

Pakdel, A., Bijma, P., Ducro, B.J. & Bovenhuis, H. (2005). Selection strategies for body weight and reduced ascites susceptibility in broilers. *Poultry Science,* 84(4), 528-535.

Patterson, R., Youngner, J.S., Weigle, W.O. & Dixon, F.J (1962). Antibody production and transfer to egg yolk in chickens. *Journal of Immunology.* 89, 272-278.

Payne, L. N., Frazier, J. A. & Powell, P. C. (1976). Pathogenesis of Marek's disease. *International Review of Experimental Pathology,* 16, 59-154.

Peckham, M. C. (1982). Poisons and toxins. In M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid & H. W. Yoder (Eds.), *Diseases of Poultry 8th Edition* (pp. 738-818). Ames, IA: Iowa State University Press.

Persico, P., Roccabianca, P., Corona, A., Vercelli, A. & Cornegliani, L. (2011). Detection of feline herpesvirus 1 via polymerase chain reaction and immunohistochemistry in cats with ulcerative facial dermatitis, eosinophilic granuloma complex reaction patterns and mosquito bite hypersensitivity. *Veterinary Dermatology,* 22, 521-527.

Phillips, P. H., & Engel, R. W. (1939). Some histopathological observations on chicks deficient in the chick antidermatitis factor in pantothenic acid. *Journal of Nutrition,* 18, 227-232.

Platt, S. R. (2006). Evaluating and treating the nervous system. In G. J. Harrison & T. L. Lightfoot (Eds.), *Clinical Avian Medicine* (pp. 493-517). Florida: Spix Publishing Inc.

Prathapkumar, S. H., Rao, V. S., Paramkishan, R. J., & Bhat, R. V. (1997). Disease outbreak in laying hens arising from the consumption of fumonisin-contaminated food. *British Poultry Science,* 38, 475-479.

Quinteiro-Filho, W.M., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M.L., Sakai, M., Sa, L.R.M., Ferreira, A.J.P. & Palermo-Neto, J. (2010). Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poultry Science,* 89(9), 1905-1914.

Ralapanawe, S., Renz, K.G., Burgess, S.K. & Walkden-Brown, S.W. (2015). Field studies on the detection, persistence and spread of the Rispens CVI988 vaccine virus and the extent of co-infection with Marek's disease virus. *Australian Veterinary Journal,* (Accepted).

Reagan, R. L., Day, W. C., Harmon, M. P., & Brueckner, A. L. (1952). Response of the baby chick to West Nile virus. *Experimental Biology and Medicine,* 80(2), 210-212.

Reece, R. (2010). Laboratory Report M10-03663. Menangle NSW: State Veterinary Diagnostic Laboratory, Elizabeth Macarthur Agricultural Institute.

Renz, K.G., Achari, R.R., Walkden-Brown, S.W. & Katz, M.E. (2015). A loop isothermal amplification assay to detect fowl adenovirus-8 in a variety of poultry materials. *Proceedings of the Australian Poultry Science Symposium* (pp. 171-175), Sydney: University of Sydney.

Renz, K.G., Cooke, J., Clarke, N., Cheetham, B.F., Hussain, Z, Islam, A.F.M., Tannock, G.A. & Walkden-Brown, S.W. (2012). Pathotyping of Australia isolates of Marek's disease virus and association of pathogenicity with meq gene polymorphism. *Avian Pathology,* 41, 161-176.

Rivers, T. M. (1937). Viruses and Koch's Postulates. *Journal of Bacteriology*, 33(1), 1-12.

Sandmeyer, L.S., Waldner, C.L., Bauer, B.S, Wen, X. & Bienzle, D. (2010). Comparison of polymerase chain reaction tests for diagnosis of feline herpesvirus, *Chlamydophila felis* and *Mycoplasma* spp. infection in cats with ocular disease in Canada. *Canadian Veterinary Journal*, 51, 629-633.

Schat, K. A., & Nair, V. K. (2008). Marek's Disease. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 452-514). Ames, Iowa: Blackwell Publishing.

Schwabe, C.W. (1984). *Veterinary Medicine & Human Health 3rd Edition.* London: Williams & Wilkins.

Senne, D. A., Pedersen, J. C., Hutto, D. L., Taylor, W. D., Schmitt, B. J., & Panigrahy, B. (2000). Pathogenicity of West Nile virus in chickens. *Avian Diseases*, 44(3), 642-649.

Sharma, J. M. & Lee, L. (1977). Suppressive effect of cyclophosphamide on the T-Cell system in chickens. *Infection and Immunity*, 17(1), 227-230.

Sharma, J.M. & Stone, H.A. (1972). Genetic resistance to Marek's disease. Delineation of the response of genetically resistant chickens to Marek's disease virus infection. *Avian Diseases,* 16, 894-906.

Shivaprasad, H. L. (2008). Arizonosis. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 665-674). Ames, Iowa: Blackwell Publishing.

Silva, R. F. (1992). Differentiation of pathogenic and non-pathogenic serotype-1 Marek's Disease Viruses (MDVs) by the polymerase chain-reaction amplification of the tandem direct repeats within the MDV genome. *Avian Diseases*, 36(3), 521-528.

Sutter, R.W., Cochi, S.L. & Melnick, J.L. (1999). Live attenuated poliovirus vaccines. In S.A. Plotkin & W.A. Orenstein (Eds.). *Vaccines (pp. 364-403).* Saunders: Philadelphia.

Swayne, D. E., Fletcher, O. J., & Schierman, L. W. (1989a). Marek's disease virus-induced transient paralysis in chickens. 1. Time course association between clinical signs and histological brain lesions. *Avian Pathology*, 18(3), 385-&.

Swayne, D. E., Fletcher, O. J., & Schierman, L. W. (1989b). Marek's disease virus-induced transient paralysis in chickens. 2. Ultrastructure of central nervous system. *Avian Pathology*, 18(3), 397-412.

Swayne, D. E., Fletcher, O. J., Tyler, D. E., & Page, R. K. (1989c). Marek's disease virus-induced transient paralysis in chickens. 3. Differentiation of field cases from classical Marek's disease by central nervous system lesions. *Avian Pathology*, 18(3), 413-431.

Swayne, D. E., & Halvorson, D. A. (2008). Influenza. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan & D. E. Swayne (Eds.), *Diseases of Poultry 12th Edition* (pp. 153-184). Ames, Iowa: Blackwell.

Thomas, R.J. (1991). Detection of Clostridium botulinum types C and D toxin by ELISA. *Australian Veterinary Journal*, 68(3), 111-113.

Thrusfield, M. V. (2005). *Veterinary Epidemiology Third Edition* (pp. 1-502). Oxford: Blackwell.

Toribio, J.A. (2008a). *Introduction to epidemiology.* Lecture notes presented in the course VETS 3025 Veterinary Public Health. University of Sydney.

Toribio, J.A. (2008b). *Study Design.* Lecture notes presented in the course VETS 3025 Veterinary Public Health. University of Sydney.

Toyoda, T., Ochiai, K., Hatai, H., Murakami, M., Ono, E., Kimura, T., & Umemura, T. (2006). Cerebellar hypoplasia associated with an avian leukosis virus inducing fowl glioma. *Veterinary Pathology*, 43(3), 294-301.

Tulman, E. R., Afonso, C. L., Lu, Z., Zsak, L., Rock, D. L. & Kutish, G. F. (2000). The genome of a very virulent Marek's disease virus. *Journal of Virology*, 74(17), 7980-7988.

Van Roekel, H., Bullis, K.L. & Clarke, M.K. (1941). Transmission of avian encephalomyelitis. *Journal of the American Veterinary Medical Association*, 99, 220.

Veen, P. J. (1973). Torticollis and disease of the respiratory tract, caused by Aspergillus fumigatus in fowl. *Netherlands Journal of Veterinary Science*, 5(2), 132-133.

Vicart, S., Sternberg, D., Arzel-Hezode, M., Franques, J., Bendahhou, S., Lory, P., Hainque, B., Fournier, E., Nicole, S. & Fontaine, B. (2014). Hypokalemic Periodic Paralysis. In R. A. Pagon, M. P. Adam, H. H. Ardinger, S. E. Wallace, A. Amemiya, L. J. Bean, T. D. Bird, C. R. Dolan, C. Fong, R. J. Smith & K. Stephens (Eds.), *GeneReviews.* Seattle: University of Washington. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK1338/.

Wajid, S.J. (2013). *Aspects of the pathogenesis and molecular epidemiology of Marek's disease in chickens* (Doctor of Philosophy), University of New England, Armidale.

Wajid, S. J., Katz, M. E., Renz, K. G. & Walkden-Brown, S. W. (2013). Prevalence of Marek's disease virus in different chicken populations in Iraq and indicative virulence based on sequence variation in the EcoRI-Q (meq) gene. *Avian Diseases*, 57, 562-568.

Walkden-Brown, S.W. (2014). *Epidemiology and control of disease.* Lecture notes presented in the course ANPR 321 Animal Production. University of New England.

Walkden-Brown, S.W., Islam, A., Islam, A.F.M.F., Burgess, S.K., Groves P.J. & Cooke, J. (2013). Pathotyping of Australian isolates of Marek's disease virus in commercial broiler

chickens vaccinated with herpesvirus of turkeys (HVT) or bivalent (HVT/SB1) vaccine and association with viral load in the spleen and feather dander. *Australian Veterinary Journal,* 91, 341-350.

Westbury, H. A. & Sinkovic, B. (1978a). The pathogenesis of infectious avian encephalomyelitis. 2. The effect of immunosuppression on the disease. *Australian Veterinary Journal,* 54(2), 72-75.

Westbury, H.A. & Sinkovic, B. (1978b). The pathogenesis of infectious avian encephalomyelitis. 4. The effect of maternal antibody on the development of disease. *Australian Veterinary Journal.* 54(2), 81-85.

White, C.L. & Taxy, J.B. (1983). Early morphologic diagnosis of herpes simplex virus encephalitis: advantages of electron microscopy and immunoperoxidase staining. *Human Pathology,* 14(2), 135-139.

Wilesmith, J.W., Wells, G.A.H., Cranwell, M.P. & Ryan, J.B.M (1988). Bovine spongiform encephalopathy: epidemiological studies. *Veterinary Record,* 123, 638-644.

Willeberg, P. (1981). Epidemiology of the feline urological syndrome. *Advances in Veterinary Science,* 25, 311-344.

Wise, D. R. (1975). Skeletal abnormalities in table poultry--a review. *Avian Pathology,* 4(1), 1-10.

Witter, R.L., Gimeno, I.M., Reed, W.M. & Bacon, L.D. (1999). An acute form of transient paralysis induced by highly virulent strains of Marek's disease virus. *Avian Diseases,* 43(4), 704-720.

Witter, R.L., Sharma, J.M. & Fadly, A.M. (1980). Pathogenicity of variant Marek's disease virus isolates in vaccinated and unvaccinated chickens. *Avian Diseases,* 24(1), 210-232.

Wood, D.E. & Salzberg, S.L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biology,* 15, R46.

Xie, Z., Khan, M. I., Girshick, T. & Xie, Z. (2005). Reverse Transcriptase-Polymerase Chain Reaction to Detect Avian Encephalomyelitis Virus. *Avian Diseases,* 49(2), 227-230.

Young, R. J., Edwards, H. M. J., & Gillis, M. B. (1958). Studies on zinc in poultry nutrition. II. Zinc requirement and deficiency symptoms of chicks. *Poultry Science,* 37, 1100-1107.

Zander, D.V. (1959). Experiences with epidemic tremor control. In: *Proceedings of the 8th Annual Western Poultry Disease Conference*. Davis, CA: 18-23.

Zerbino, D.R. & Birney, E. (2008). Velvet: Algorithms for de novo short read assembly using de Brujin graphs. *Genome Research,* 18(5), 821-829.

Zhang, T., Wang, B., Ling, Z., Li, Y., Gao, X., Tian, G., Zhang, J., Han, L. & Zhang, L. (2009). *Chinese Journal of Animal Quarantine,* 26, 58-60.

Zhang, G., Zhang, Y., Kim, Y., Kim, J., Liu, L., Yu, X. & Teng, X. (2011). Field study on the impact of indoor air quality on broiler production. *Indoor and Built Environment, 20(4), 449-455.*