THE EFFECT OF ENVIRONMENTAL STRESSORS ON THE IMMUNE RESPONSE TO AVIAN INFECTIOUS BRONCHITIS VIRUS

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THE EFFECT OF ENVIRONMENTAL STRESSORS ON THE IMMUNE RESPONSE TO AVIAN INFECTIOUS BRONCHITIS VIRUS (IBV)

by Juan Carlos Lopez

The first aim of this research was to determine the prevalence of IBV in broilers within the Canterbury province, New Zealand, in late winter and to search for associations with management or environmental factors. The second aim was to study how ambient stressors affect the immune system in birds, their adaptive capacity to respond, and the price that they have to pay in order to return to homeostasis.

In a case control study, binary logistic regression analyses were used to seek associations between the presence of IBV in broilers and various risk factors that had been linked in other studies to the presence of different avian pathogens: ambient ammonia, oxygen, carbon dioxide, humidity and litter humidity. Pairs of sheds were selected from ten large broiler farms in Canterbury. One shed (case) from each pair contained poultry that had a production or health alteration that suggested the presence of IBV and the other was a control shed. Overall, IBV was detected by RT-PCR in 50% of the farms. In 2 of the 5 positive farms (but none of the control sheds) where IBV was detected there were accompanying clinical signs that suggested infectious bronchitis (IB). Ambient humidity was the only risk factor that showed an association (inverse) with the prevalence of IBV ($p = 0.05$; OR = 0.92). It was concluded within the constraints of the totally enclosed management systems described, that humidity had an influence on the presence of IBV, but temperature, ammonia, carbon dioxide, oxygen or litter humidity had no effect.

In another study environmental temperatures were changed in order to affect the biological function and adaptive capacity of chickens following infection with IBV. The 'affective states' of the animal were assessed by measuring levels of corticosterone (CORT) in plasma and tonic immobility (TI). It was found that low (10 +/- 2°C) and high (30 +/- 2°C) temperatures exacerbated the respiratory signs and lesions in birds infected with IBV as compared to those housed at moderate (20 +/- 2°C) temperatures. The chickens housed at high temperatures showed significantly decreased growth, a higher proportion of hepatic lesions (principally haemorrhages) and a longer tonic immobility period, but there was no significant alteration in the plasma levels of CORT. The birds housed at low temperatures developed a higher proportion of heart lesions (hydropericardium, ventricular hypertrophy) and had significantly higher levels of plasma CORT than birds housed under moderate and/or high temperatures. The specific antibody response to IBV decreased in birds housed under high temperatures. Interestingly the birds housed at high temperatures developed significantly higher levels of haemagglutinin antibodies to sheep red blood cells (SRBC) than those birds housed under low or moderated temperatures. Cell mediated immunity was not significantly affected by heat or cold stress in the first 13 days of treatment but at 20 days the levels of interferon gamma in the birds subjected to low temperatures were lower than in the high temperature group.

In other trials, the exogenous administration of low physiological doses of oral CORT (as compared to high pharmacological doses typically used in such experiments) to birds resulted in suppression or enhancement of the immune response depending on duration of treatment and/or dose and nature of the antigen. To our knowledge, this is the first study to show that exogenous CORT can produce an enhancement in the immune response in chickens.

In conclusion, environmental stressors such as high or low temperatures do affect the physiology of the fast-growing broiler. The adjustments the birds have to make to maintain homeostasis impacts on the course of common infectious diseases, such as IB, that normally is mild in the New Zealand poultry industry. The administration of exogenous CORT showed that this hormone may be part of the physiological stress response and acts as a messenger to prepare the immune system for potential challenges (e.g., infection).

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Abbreviations

ACTH	Adrenocorticotropic hormone
AGPT	Agar gel precipitation test
ASC	Antibody secreting cells
AVT	Arginine vasotocin
CKC	Chicken kidney cells
CI	Confidence interval
CMI	Cell mediated immunity
ConA	Concanavalin A
CORT	Corticosterone
CRF	Corticotropin releasing factor
CTL	Cytotoxic T lymphocyte
ELISA	Enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
GAS	General adaptation syndrome
GC	Glucocorticoid
HI	Haemagglutination inhibition
HPA	Hypothalamo pituitary adrenal axis
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
IFA	Immunofluorescence assay
IL	Interleukin
INF	Interferon
IPA	Immunoperoxidase assay
KLH	Keyhole limpet haemocyanin
MAF	Ministry of Agriculture and Forestry
MDA	Maternal derived antibody

MG	Mycoplasma gallisepticum
MHC	Major histocompatibility complex
NK	Natural Killer
OR	Odds ratio
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PCV	Packed cell volume
PHA	Phytohaemagglutinin
PHS	Pulmonary hypertension syndrome
rChIFN	Recombinant chicken interferon
RIA	Radioimmunoasaay
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RVH	Right ventricular hypertrophy
SEM	Standard error of the mean
SRBC	Sheep red blood cells
TI	Tonic immobility
TM	Transport media
TNF	Tumour necrosis factor
VI	Virus isolation
VN	Virus neutralisation

Chapter 1

THE EFFECT OF ENVIRONMENTAL STRESSORS ON THE IMMUNE RESPONSE TO AVIAN INFECTIOUS BRONCHITIS VIRUS (IBV)

INTRODUCTION

The importance of poultry production has increased during recent decades and will increase in the future due to the rise in demand for food, together with human population growth. Almost every country in the world has a poultry industry of some kind. According to the Food and Agriculture Organization of the United Nations (FAO) poultry meat is ranked as the second major source of protein for the world's population behind pork. In many countries poultry is the meat with the highest per capita consumption (e.g., USA, New Zealand).

The FAO predicted a 2.9 percent per year increase in demand for poultry meat around the world until the end of 2015, against a 1.4 annual percent increase in demand annually for beef. Although the poultry industry is relatively new in New Zealand, it has been expanding rapidly. According to the Poultry Industry Association of New Zealand, in 2004, the poultry industry produced more than 87.5 million broiler chickens, weighing 151,497 tonnes. Poultry meat annual consumption continues to increase in New Zealand, up from 14 kg per capita in 1986, to 37.10 kg per capita in 2006. In addition, New Zealand currently has 2.7-2.8 million laying hens that produce around 816-880 million eggs.

Despite progress that has been made in the areas of health, vaccine use and management in the poultry industry, problem diseases still exist that affect the efficiency of the poultry industry. Seasonal cycles of infectious diseases have been variously attributed to changes in environmental conditions, the prevalence or virulence of the pathogen, or the behaviour of the host, but no single theory has proved a satisfactory explanation (Dowell 2001). Avian infectious bronchitis virus (IBV) is a major respiratory virus of chickens, as it is probably

endemic in all countries that raise chickens (Cavanagh and Naqi 2003). Diseases caused by coronaviruses such as IBV are more frequent in winter, with a unique ability to establish persistent infections in a minority of infected animals (Holmes 2003; Dowell and Ho 2004).

In the majority of the more affluent countries, broilers are reared intensively in environmentally controlled sheds, without cages, and feed and water are available *ad libitum*. However, some farmers in developed countries have returned to old fashioned 'free range chicken' rearing methods because of demands from a sector of consumers with a desire for natural or organic food products (Darre 2003). Under such farming systems the birds face pressure from different pathogens and, simultaneously, undergo various kinds of physical, climatic and social stresses (Hangalapura et al 2003; Van Loon et al 2004) of a different type from those experienced in intensive housing.

In New Zealand farmers use broiler chicken populations that have been genetically selected for rapid growth. These modern breeds of chickens have been bred for confined conditions and require precise environmental control to achieve maximum performance (Darre 2003). Studies comparing the red jungle fowl (one predecessor of modern poultry breeds) to commercial layer and broiler chickens have documented the reduced respiratory capacity of the fast-growing chicken (Vidyadaran et al 1987).

The study of animal welfare continues to struggle with two persistent problems: how to define animal welfare and how to determine which measures should be used to evaluate welfare (Mench 2000). One common indicator used for the determination of animal welfare is the presence of stress. The perception of the general public is that any management or condition that causes stress for the animals should be avoided or prohibited. One scientific point of view, however, is that stress endangers the animal's welfare only if it results in some significant biological change that places that animal's health at risk (Moberg 2000). The term

'distress' has been used by some researchers in order to differentiate between a non-threatening stress response ('good stress') and the biological stage where the response has a deleterious effect on the animal's welfare. Measurements of immune competence offer us a potentially powerful tool for evaluating the results of distress and for differentiating between stress and distress situations (Moberg 2000). It is widely assumed that the negative economic impact of disease is enhanced in distressed animals (Dohms and Metz 1991), and this may be mediated via a neuroendocrine response that affects the normal performance of the immune organs (El-Lethey et al 2003).

Some factors are believed to intensify the clinical signs and lesions present in various diseases or affect the immune response in poultry: low or high environmental temperatures (Regnier and Kelly 1981; Ratanasethakul and Cumming 1983; Henken et al 1983; Hangalapura et al 2003; Hangalapura et al 2004a) high ammonia levels (Anderson et al 1964; Kling and Quarles 1974), low levels of oxygen (Olander et al 1967) and circulating corticosterone levels (El-Lethey et al 2003). Supporting evidence from studies that have been trying to explain the effect of stress on the avian immune system are based, to a large extent, on the use of non-natural stress models (El-Lethey et al 2003). Moreover, in the majority of these studies, the humoral response to a single antigen or pathogen was considered, but the impact of stress upon cell-mediated immunity has not been addressed (El-Lethey et al 2003). Only some of these factors have been studied in relation to immunity following an IBV infection. Welfare is a complex phenomenon and only a multifactorial approach, including physical health, production traits and physiological and behavioural indicators, can allow a relevant assessment (Neindre et al 2004).

The first aim of this research was to determine the prevalence of IBV in broilers within the Canterbury province in late winter and search for associations with managemental or environmental factors. The second aim of this research was to study how ambient stressors affect the immune system and adaptive capacity of broilers.

Chapter 2

LITERATURE REVIEW

2.1 Seasonal cycles of infectious diseases

Disease is the result of complex interactions (some would say imbalance) between the triad of the agent (toxic or infectious), the host and the environment (Campbell et al 1983). Many diseases show a regular and predictable pattern of seasonality. Different infections peak in each of the four seasons, but each pathogen and the timing and characteristics of the annual outbreak are remarkably consistent from year to year (Dowell 2001). Diseases caused by coronaviruses such as IBV, show winter seasonality (Holmes 2003). The origin of this seasonality is not clear. It can be attributed to environmental changes, changes in host physiology or alterations in the virus (Dowell 2001). Ramneek et al (2005) showed that the sequences of the S1 gene isolated from historic and new field isolates of IBV in New Zealand had a high homology indicating very little season to season change. Seasonal changes in host physiology are controlled principally by the duration of the night/dark cycle and mediated by changes in the daily melatonin pulse (Dowell 2001). The majority of the poultry sheds in New Zealand are closed, with a similar light/dark programme throughout the whole year. Hence it is expected that only ‘free range’ poultry would be affected by such mechanisms.

2.2 Welfare and stress in poultry

Since the controversy generated by Harrison (1964) with the publication of her book *Animal Machines: The New Factory Farming Industry*, there has been an enormous debate over what exactly animal welfare is and how it should be assessed. Duncan and Dawkins (1983) and Broom (1986) reviewed the definitions of welfare that various investigators had proposed and concluded that it is impossible to give welfare a precise scientific definition and that the best that can be achieved is a broad working description encircling the ideas of the animal’s

physical and mental health; the animal being able to adapt to its environment without suffering. Diseases are a major and persistent factor in animal suffering, and combating them must be a priority for all those seeking to improve animal welfare [Office International des Epizooties (OIE) 2005].

Society has divergent views with regard to how animal welfare should be assessed (Wyss et al 2004). In general, there are three different ways that welfare has been measured: 'biological functioning', 'affective states' and 'natural living' (Fraser 2004). According to 'biological functioning', good animal welfare is characterised by a high level of health, growth, production efficiency and correlated traits (Fraser 2004). According to the second point of view, animal welfare should focus on the way the animal perceives its environment in terms of emotions (such as fear and pain) which is the ultimate goal of the assessment ('affective states') (Neindre et al 2004). Duncan et al (1986) used the tonic immobility test in chickens in order to assess animal welfare from the 'affective states' point of view. They found that the mechanical harvester system produced less fear in the chickens than the traditional manual catching system. The third position 'natural living' believes that animals should be allowed to lead reasonably natural lives by carrying out their normal behaviour, free from restraint. The majority of current welfare audit schemes (e.g., www.biosecurity.govt.nz/animal-welfare) are based on standards set around prescribing the resources available to the bird, such as litter type or ventilation system capacity 'input measures'. Many authors consider that input measures are much less reliable as welfare assessment measures than 'output' measures; that is, those based on the 'experience' of the bird (Moberg 2000).

Each of these viewpoints make valid claims and attracts valid criticism (Fraser 2004). One common indicator used for the determination of animal welfare is the presence of stress. Fraser et al (1975) defined stress in a veterinary context as 'an abnormal or extreme

adjustment in the physiology of the animal to cope with adverse effects in its environment and management'.

Since the times of Hippocrates (460-375 BC) it has been postulated that for the correct function of the body (health) it has to be in 'balance,' and when there are alterations in that balance 'disharmony' or disease occurs (Chrousos et al 1988). Stress is only negative when it adversely affects the animal's welfare, when it may be termed 'distress' (Moberg 2000). An important characteristic of stress is its duration and magnitude. It has been reported that *mild or brief stress* (minutes to hours) may enhance the cell mediated (Blecha et al 1982), humoral (Solomon 1969), and innate (Deak et al 1997) immune responses. The benefit to the host of these physiological responses is not clear, but it has been suggested that altered recirculation patterns represent a realignment of cellular duties from 'patrol' to 'battle stations' in response to an anticipated immunological challenge that would accompany injury. In contrast, *severe* stress has been shown to suppress various immune parameters (Subba Rao and Glick 1970; Ader et al 2001; El-Lethey et al 2003).

2.2.1 Mechanisms of the general adaptation syndrome (GAS)

Selye (1949) believed that animals must respond to changing environmental conditions in order to maintain homeostasis, using a non-specific stress response pathway, the GAS. Animals, after initial perception of an attack, harm or threat to their survival, mount an emergency 'alarm' phase or fight-flight response (Cannon 1914; 1932). These catecholamine-driven reactions result in increased cardiovascular function and an overall increase in metabolism. The animals need all the energy that they can afford for fight, or if the predator is 'too big', flight. If the stressor persists, the 'resistance' phase is initiated, which is a physiological coping reaction and eventually an 'exhaustion' phase may develop leading to pathology (Selye 1949; 1976; Cannon 1932; Moberg 1985). These responses may be

quantified by measurement of glucocorticoids. These hormones are released when the hypothalamo-pituitary-adrenal axis (HPA) is activated in response to a stress situation. However, many researchers believe that the GAS theory is too simplistic (Dohms and Metz 1991) as not all animals under stress produce glucocorticoids (Moberg 1985). The response may or may not differ between stressors (El-Lethey et al 2003). For example, stallions secreted similar amounts of cortisol whether they were restrained, exercised or mated with mares (Colborn et al 1991).

2.2.2 Hypothalamo-pituitary-adrenal axis (HPA)

In birds, the HPA axis is well characterized (Holmes 1978; Harvey and Hall 1990). When a stressor is perceived hypothalamic corticotropin-releasing factor (CRF) and arginine vasotocin (AVT) are secreted, which stimulate the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) (Kovacs and Peczely 1991). Circulating ACTH levels peak within 5-10 minutes of the stress (Kovacs and Peczely 1991). The release of ACTH is antagonized by hypothalamic somatostatin (SRIF) (Cheung et al 1988). Circulating ACTH in turn causes the adrenal cortex to produce glucocorticoids (GC) within 5 minutes, and in a dose-dependent manner (Radke et al 1985; Beuving and Vonder 1986). Chickens treated with ACTH (50 IU/animal) show an increase of plasma corticosterone (CORT) (the principal corticosteroid) to about 25ng/ml within 1h, followed by a drop to pre-treatment levels after 4h (Assenmacher 1973; Dehnhard et al 2003). Basal levels of CORT in one-week-old domestic chicks are approximately 11-23 ng/ml across various domestic fowl strains (Carsia and Weber 1986; Decuypere et al 1989) with basal levels decreasing as a function of age: 5-7 ng/ml in 3 weeks old commercial broilers (Post et al 2003) and to 0.42- 6.04 ng/ml in 51 week old laying hens (Littin and Cockrem 2001). In chickens, most circulating corticosterone is transported bound in dynamic equilibrium to plasma proteins (Carsia and Harvey 2000). Alterations in the

corticosterone levels in response to a variety of environmental stressors have been reported in birds (Edens and Siegel 1975; El Halawani et al 1973; Hangalapura et al 2004b). The effects of exogenous glucocorticoids are not constant and depend on the molecular species of glucocorticoid employed, the route and the time of administration (Joseph and Ramachandran 1993; Sapolsky et al 2000). Glucocorticoids alter several metabolic and immunological processes. The pre-eminent effect of glucocorticoids upon metabolism is their ability to increase circulating glucose concentrations. This is accomplished through a number of mechanisms including stimulation of appetite and gastrointestinal transit time (Santana et al 1995; Tur et al 1989). Despite the increase in food intake, there is a remarkable decrease in growth and body weight gain in chickens (Hayashi et al 1994; El-Lethey et al 2001), principally due to an increase in net muscle protein catabolism (Hayashi et al 1994; Sapolsky et al 2000). Corticosterone also stimulates gluconeogenesis (particularly in the liver), mobilization of amino acids from extrahepatic tissues, inhibition of glucose uptake in muscle and adipose tissue, and stimulation of fat breakdown in adipose tissue (Siegel and Van Kampen 1984).

Glucocorticoids can cause suppression of immune functions e.g. suppression of MHC-II expression, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-12 (Griffin 1989; Sapolsky et al 2000), TNFα and interferon gamma production (Isobe and Lillehoj, 1992, 1993). Daynes and Araneo (1989) reported that exogenous glucocorticoids suppress Th2 responses but enhance Th1 responses in mice. However, other investigators have reported enhancement of the immune functions: stimulation of immunoglobulin synthesis by cultured B cells (Cupps et al 1985), T cell responses (Wiegers et al 1993; 1994) and TNFα and IL-6 production (Barber et al 1993). Claman (1988) showed that not all animal species are impacted equally by corticosteroids, some are more sensitive (e.g., chicken, mouse, rat, rabbit and hamster) than others (e.g., primates, guinea pig, sheep, cattle, pig).

2.3 Body temperature and avian thermoregulation

The chicken, like mammals, is a homeotherm, in that it maintains a relatively constant body temperature over a wide range of environmental conditions (Freeman 1971). In adult chickens, the body temperature varies between 41°C and 42°C (Brake and Thaxton 1979). Under normal conditions the temperature of the bird is above that of the environment and, as a consequence heat is constantly being lost to the environment by a combination of radiation, conduction, convection and evaporation (Freeman 1971). As the ambient temperature rises the respiratory rate increases and at around 29.4°C (ambient temperature) when the body temperature reaches 42-42.5°C the birds begin to pant (or exhibit thermal polypnea) (Frankel et al 1962; North and Bell 1990). When this response (panting) is prolonged the bird may experience respiratory alkalosis, due to excess loss of CO_2 from the body (Borges et al 2004). In addition to panting, the birds assume unusual physical positions or change behaviour in order to help heat loss e.g. by standing with the neck extended and wings held away from the body and by effecting vasodilation in the naked parts of the body so that the heat can be dissipated from these areas (Freeman 1971). The upper lethal body temperature is 45-47°C (Randall 1943). Conversely, when the ambient temperature is low, and the heat loss exceeds heat production, the birds increase their catabolic rate in order to prevent a fall in core temperature. The feathers are fluffed out to increase insulatory protection and the birds will crowd together to reduce the effective surface area presented to the environment (Freeman 1971; Dawson and Whittow 2000). They will also sit so that the heat loss from the unfeathered portions of the legs is reduced with the head tucked under the wing (Freeman 1971). The lower lethal body temperature is approximately 23°C.

2.3.1 Physiological changes due to effect of heat or cold

Chickens subjected to high environmental temperatures exhibit many physiological and behavioural changes which allow them to re-establish a heat balance with their surroundings. One of the primary effects of high environmental temperature on a flock is a reduced feed (and energy) intake, leading to depressed growth rates (Wilson 1948; Vo et al 1978; Suk and Washburn 1995). Above the upper thermoneutral level, energy demands rise to power the active cooling mechanisms used by the bird but because appetite is reduced by the elevated temperatures health problems ensue. Darre and Harrison (1987) reported that heat distress caused blood glucose concentration to increase. Borges et al (2004) reported that heat stress alters the proportion of heterophils (increased) to lymphocytes (decreased) in blood and decreased hematocrit values occur (Deaton et al 1969; Kubena et al 1972; Vo et al 1978).

Haemoconcentration is associated with lower environmental temperatures (7°C versus 22 or 32°C) (Huston 1960; Deaton et al 1969). Blood pressure is elevated during winter months and lowered during summer months (Rosenthal 2004; Ulmer et al 2004).

Cold weather has been reported as an important factor that triggers the onset of ascites in chickens. The most frequent cause of ascites in broilers is increased portal pressure, secondary to right ventricular failure (RVF), resulting in liver damage although decreased osmotic pressure and increased vascular permeability also occur (Julian 2005). Hypothermia causes increased oxygen demand with an associated increase in cardiac output. Because the lung volume and cardiovascular volume within the lung tissue is fixed, there comes a point at which the lung can no longer accommodate any more blood being supplied by the heart and pulmonary hypertension may result. This is the starting point for heart failure (Julian 2005). With prolonged hypoxia, the body increases the oxygen carrying capacity in the blood by increasing the number of erythrocytes (polycythaemia), leading to increases in the blood

viscosity that aggravates the pulmonary hypertension (Burton and Smith 1967). Chronic hypertension results in right ventricular hypertrophy (RVH) and causes a malfunction of the right atriventricular valve, allowing blood to flow backwards into the vena cava. This leads to liver congestion and seepage of liquid from the liver surface. When the rate of leakage is greater than the capacity of the abdominal membranes to absorb the liquid, ascites develops (Julian et al 1987).

The more liquid portion of the fluid in the hepato-peritoneal spaces can return to the vena cava via the lymphatics and thoracic duct until venous pressure caused by RVH becomes too high (Julian 2005). Affected broilers become dull, depressed, inactive, cyanotic and may be in respiratory distress (Julian 2005).

2.3.2 Effect of temperature on the immune system in birds

In homeotherms not housed in a controlled environment during the winter and summer seasons, thermo-regulation is an important energy-demanding process and therefore may compete with immune function for nutrients (Hangalapura et al 2003). In wild birds, energy-demanding processes such as breeding, brood care and migration, are usually accompanied by decreased immune response and decreased health status, especially during cold seasons (Lochmiller and Deerenberg 2000). In chickens, results of the few studies on the effect of cold or hot stress on antibody-mediated and cell-mediated immune responses are not consistent (Hangalapura et al 2003). Heller et al (1979) reported that when birds were exposed to heat stress the immune response (antibody production) was enhanced. On the other hand, there are studies by Subba Rao and Glick (1970), and Mashaly et al (2004) which showed the immunosuppressive effect of heat stress in chickens.

Cold exposure has been reported to enhance (Subba Rao and Glick 1977) or decrease the immune response (Regnier and Kelly 1981). These differences in results can be attributed, in

part, to the way in which specific immunologic parameters were measured (humoral or cellular cell immunity), or the different assay techniques and chicken breeds (Subba Rao and Glick 1977; Heller et al 1979; Regnier and Kelly 1981; Donker et al 1990). Additionally, El-Lethey et al (2003) showed that depending on the antigen tested, there are stress-resistant and stress-susceptible antigen responses. They reported that chronic stress strongly suppressed humoral and cellular immune responses assessed by lower antibody titres to sheep red blood cells and tetanus toxoid but, in contrast, the antibody response to human serum albumin was neither influenced by corticosterone feeding nor by depriving the birds of foraging material (litter).

2.4 Infectious bronchitis

Infectious bronchitis (IB) is a highly contagious viral respiratory disease of chickens characterized by tracheal rales, coughing and sneezing (Schalk and Hawn 1931) and in some cases, renal pathology (Cumming 1962). In laying flocks there is typically a drop in egg production and egg quality (Sevoian and Levine 1957). In meat type chickens, IB decreases live weight gain and feed efficiency as a component of mixed infections (Cavanagh and Naqi 1997). Losses from production inefficiencies are usually of greater concern than losses from mortality, particularly in New Zealand where concurrent infections from immunosuppressive agents such as infection bursal disease virus (Chai et al 2001) and chicken anaemia virus (Stanislawek and Howell 1994; Markowski-Grimsrud and Schat 2003) are uncommon.

Some researchers have postulated that the infectious bronchitis virus (IBV) is a pathogen that has been present in the birds since the first jungle fowl (four to eight thousand years ago), and viral changes are linked to the domestication process (Cavanagh 2002). The first description of IB was published by Schalk and Hawn in 1931. At that time, IB was recognized primarily as a disease affecting young chicks, but years later reductions in egg production and quality

attributed to IBV, were reported by Van Roekel et al (1951) and Broadfoot and Smith (1954). In 1936, Beach and Schalm established the viral aetiology, and later on, Beaudette and Hudson (1937) made the first cultivation of the virus in embryonated chickens' eggs. At that time, it was assumed that all IBV strains were similar antigenically, but in 1956 Jungherr and colleagues established that the Connecticut and Massachusetts isolates produced similar clinical signs and lesions but did not cross-protect or cross-neutralize. In 1962, Cumming reported that IBV was the cause of the nephrosis-nephritis syndrome seen in Australia. The disease has been reported in broilers, layers and breeder chickens throughout the world: Europe (Asplin 1948), South America (Garcia and Norambuena 1969), Asia (Sato et al 1955), Africa (El Houadfi et al 1986) and Oceania (Cumming 1963). Outbreaks of IB frequently occur, even in vaccinated flocks (Cavanagh and Naqi 2003).

2.4.1 IB in New Zealand

IBV was first isolated in New Zealand by Pohl in 1967. These initial isolates were shown to be serologically different from strains from USA, Australia, and Germany (Bülow 1969). IBV was isolated again in New Zealand in 1972, by McCausland et al, at which time IB was considered an infrequent, mild illness. Soon after, a study showed that about 75% of the broiler and layer flocks had antibodies to IB, even though vaccination was not practised in New Zealand at that time (Lohr 1974). From 1972 to 1976 four different serotypes of IBV were isolated, and named A, B, C, and D and Lohr (1977) showed serological associations with strains from Australia, USA and Europe (Lohr 1976; Lohr and Locher 1983). A high prevalence of IBV (19%) has been demonstrated recently in layers and broilers using a reverse transcriptase polymerase chain reaction (RT-PCR) (Ramneek et al 2005). At the end of the 1970's a commercial vaccine was derived from the New Zealand A field strain by serial passage in embryonated eggs (Lohr 1977). This vaccine is still the only live vaccine available

in New Zealand and Ramneek (2000) found that it gave only partial protection to recent field isolates. Ramneek et al (2005) and McFarlane et al (2002) found that there were no major genetic differences between the historical strains A, B, C and D and new (1996-2000) field isolates; 23 out of 28 isolates had >92% homology with the historical strains B,C and D and the remainder were similar to A. When the phylogenetic relationship between the New Zealand and other international (European, North American, Chilean, and Asian) strains was analysed, the former were generally most closely related to the Australian strain (Group 1). Indeed, the New Zealand A strain shared 99% of nucleotide and 98% amino acid homology with the Australian Vic S (vaccine) and V5/90 strains (Ramneek 2000; Ramneek et al 2005).

2.4.2 Aetiology

IB is caused by avian infectious bronchitis virus (IBV), a member of the Family *Coronaviridae*, Genus *Coronavirus*. The morphology is described as pleomorphic, enveloped, 80-120 nm in diameter, surrounded by a corona of club-shape projections or spikes about 20 nm in length (Berry et al 1964). IBV virions enclose three main structural proteins: the spike (S), membrane (M) and internal nucleoprotein (N) (Lai and Cavanagh 1997). Also, a small membrane protein (E) is believed to be connected with the virion envelope (Cavanagh and Naqi 2003). The S protein comprises two or three copies of each of two glycopolypeptides, S1 and S2. S1 is responsible for hemagglutination-inhibition (HI) and most of the virus-neutralizing antibodies (Cavanagh et al 1988).

2.4.3 Strain classification

Classification of strains is complicated by the lack of a standard worldwide test, the use of different names for the same type of virus, the number of diverse test systems available and, perhaps most importantly, the nature of this virus (De Wit 2000). Classification systems can be separated into two main groups: functional tests, which look upon the biological function

of a virus (immuno- or protectotypes, serotypes and epitope-types) and non-functional tests (genotypes) (De Wit 2000). The grouping of IBV into immunotypes (Cunningham 1975) or protectotypes (Lohr 1988) provides direct information about the efficacy of vaccine and strains that induce protection against each other belong to the same immuno- or protectotype (De Wit 2000). The estimation of protectotypes requires a cross-immunization study (CIS) where vaccinated birds are challenged with different strains, which is laborious, expensive and requires many animals and isolation facilities (Cook et al 1999a; Gelb et al 1981; Gelb et al 1991). Dhinakar Raj and Jones (1996b) used a more economical *in vitro* alternative for the CIS; the cross-inmunization test (CIT) where the challenge is performed on tracheal organ cultures dissected from vaccinated chickens.

In the past, classification by antigenic types has been the most widely used classification, and is based on the reaction between an IBV strain and IBV serotype-specific antibodies raised in chickens. Dozens of serotypes of IBV have been detected; without doubt, there are many more (Cavanagh and Naqi 2003). The technique most frequently used is virus neutralization (VN) but HI (Lashgari and Newman 1984; Cook et al 1987) and reactivity with panels of monoclonal antibodies is also practiced (Ignjatovic and McWaters 1991). Recently the grouping of strains based on genetic characterization has become more common (De Wit 2000). Methods commonly used include sequencing (Andreasen et al 1991; Zwaagstra et al 1992; Ramneek 2000; Lopez et al 2006), genotype-specific RT-PCR (Keeler et al 1998), restriction enzyme fragment length polymorphism (Lin et al 1991; Adzhar et al 1996; Jackwood et al 1997; Escorcia et al 2000), and hybridization (Jackwood et al 1992; Kwon et al 1993). Several studies have reported a high similarity between the genotype and serotype of certain strains (Keeler et al 1998; Lin et al 1991; Ramnnek 2000), whereas other studies do not (Capua et al 1998; Kusters et al 1987).

The majority of the IBV serotypes differ from each other by 20 to 25% of the S1 amino acids (Adzhar et al 1997; Kingham et al 2000), although several differ by up to 50% (Cavanagh et al 1997, Gelb et al 1997). In New Zealand, the deduced amino acid sequences of the S1 protein differed by 1 – 23% between isolates (Ramneek et al 2005). The differences between other IBV proteins do not usually exceed 15% (Cavanagh et al 2001).

2.4.4 Transmission and carriers

IBV affects chickens of all ages, and they are considered to be the only host. Genetically, IBV is very similar to the coronavirus which causes enteric disease in turkeys (Cavanagh et al 2001), and respiratory and kidney diseases in pheasants (Gough et al 1996). Evidence is increasing that IBV has a wider host range than was previously thought e.g peafowl and teal (Liu et al 2005). The respiratory route of infection is by droplets expelled by coughing and or sneezing by infected chickens. It has been reported that a strain that has been not associated with nephrosis, can cause kidney lesions when inoculated intra-cloacally (Uenaka et al 1998). Susceptible birds reared together with infected chickens generally develop signs of illness within 48 hours. The transmission from farm to farm is, principally, by the movement of birds, contaminated equipment, people and vehicles. After a bird is infected, it carries and sheds the virus for a week or longer (Ramneek 2000). The virus has been isolated from eggs and semen (Cook 1971) but vertical transmission has not been demonstrated.

2.4.5 Clinical signs

All ages are susceptible, but the clinical signs are more severe in young chicks (Animas et al 1994). The initial replication of IBV in the respiratory tissue causes typical, but not pathognomonic, signs such as breathlessness, coughing, sneezing, tracheal rales and nasal discharge (Dhinakar Raj and Jones 1997a). Inflamed eyes and swollen sinuses have also been reported (Parsons et al 1992; Capua et al 1994). In chicks, feed intake and live weight gain

can be reduced (Cavanagh and Naqi 2003). Hofstad (1984) reported that in animals older than six weeks, nasal discharge does not occur as frequently, and the disease may go unobserved unless the birds are examined carefully either by handling the birds or listening to them at night when the birds are normally quiet. In layers, a decline in egg production and quality is seen with or with out respiratory signs (Sevoian and Levine 1957). Broiler chickens infected with a nephropathic strain after recovering from the typical respiratory phase may then show signs of depression, ruffled feathers, wet droppings and increased water intake (Winterfield and Hitchner 1962; Cumming 1969).

Generally, mortality is low if the disease is not complicated by other factors such as the infectious agents e.g., *M. gallisepticum*, *E. coli,* immunosuppression or environmental stressors such as ammonia or changes in environmental temperature (Ratanasethakul and Cumming 1983; Peighambari et al 2000). As age increases chicks become more resistant to mortality (Crinion and Hofstad 1972; Albassam et al 1986). Infection with IBV may predispose chickens to secondary infections such as *Esherichia coli* (reviewed by Dhinakar and Jones 1997a) or *Ornithobacterium rhinotracheale* (not present in New Zealand), which may bring about increased condemnation at the processing plant, especially when infection occurs in the last weeks prior to slaughter

In New Zealand, uncomplicated infections with IBV are mild under ideal environmental conditions (Ramneek 2000) and disease outbreaks occur when there are changes in certain housing and management practices, even in vaccinated flocks (Findon 1987).

2.4.6 Pathogenesis

IBV initially infects the upper respiratory tract, where it is restricted to the ciliated and mucus-secreting cells (Dhinaker Raj and Jones 1997a). The virus titres are maximal in the nose and trachea within three days of infection and remain so for two to five days (Hostad and

Yoder 1966). Small areas of pneumonia may be observed in the lungs, although IBV is not considered to cause pneumonia (Dhinaker Raj and Jones 1997a). Deciliation of the ciliated epithelia of the nasal passages and trachea follows infection. Infection is commonly followed by secondary bacterial infections (e.g., *Escherichia. coli*), which can be the main cause of debilitating disease (Vandekerchove et al 2004). Subsequent to the initial viral replication in the epithelial cells of the respiratory tract, a viremia occurs and the virus is widely circulated to other tissues such as kidney, oviducts, testes, oesophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, caecal tonsils, rectum and cloaca (Jones and Jordan 1971; McMartin 1993; Dhinaker Raj and Jones 1997a). IBV can establish persistent infections in chickens (e.g., kidney and caecal tonsils). Virus may not be detectable after the initial period of replication, but may be excreted at the beginning of egg production (Jones and Ambali 1987).

2.4.7 Lesions

Infected chickens have accumulations of serous, catarrhal or caseous exudate in the tracheal, nasal passages and sinuses. If complicating factors are present air sacs may appear cloudy or contain a yellow caseous exudate (Cavanagh and Naqi 2003). In laying hens the virus causes glandular hypoplasia in the oviduct that leads to reduction in the synthesis of albumin proteins, especially ovomucin, lysozyme and other major proteins which constitute the structural matrix of the thick albumen, and lead to ‘watery-whites’(Butler et al 1972). Effects of IBV on the male reproductive tract have not been reported (Dhinakar Raj and Jones 1997a).

After infection with nephropathogenic strains, the kidneys become pale and swollen and urate deposits may form in the kidneys and ureters (Cumming 1963). The virus causes granular degeneration, vacuolation and desquamation of the tubular epithelium with massive

infiltration of heterophils in the interstitium in acute stages of the disease (Chen et al 1996) which may lead to wet droppings. Gough et al (1992) reported that the strain 793/B was isolated from broiler breeding flocks with bilateral myopathy in both deep and superficial pectoral muscles. Even though IBV has a wide tropism for gut tissues, no gross or histological changes have been reported (Dhinakar Raj and Jones 1997a).

2.4.8 Diagnosis

IBV infections can be diagnosed by detection of the virus itself or part of it, or a specific antibody response (De Wit 2000). Traditionally, virus isolation (VI) is the first step in the detection of IBV. The trachea is a primary target for IBV, and so is usually chosen as a sampling site, especially within the first week of infection (McMartin 1993; Dhinakar Raj and Jones 1997a; Cavanagh and Naqi 2003). Ambali and Jones (1990) found that the highest virus titres are reached in the trachea between five to ten days p.i. One week after clinical signs have appeared, the possibilities of isolating IBV are higher in cloacal swabs or caecal tonsils, kidney or oviduct than in the trachea (Cook 1968; Alexander and Gough 1977; El-Houadfi et al 1986; Ramneek 2000; Cavanagh and Naqi 2003). IBV can be isolated in different biological systems such as embryonated eggs (Jordan and Nassar 1973) and chicken tracheal organ cultures (Darbyshire 1978). VI is a sensitive technique but can be laborious, time consuming (should be passaged through embryonated eggs or tracheal organs several times for adaptation) and costly (De Wit 2000). Also, VI observations are not, in themselves, sufficient to confirm the presence of IBV. The presence of the virus must then be confirmed by ELISA, immunohistochemistry, nucleic acid analysis, or electron microscopy. There may be circumstances when VI fails, for example when IBV is inactivated (high temperature) or a double infection with IBV and another virus occurs. In such instances these techniques, or

others such as agar-gel precipitation test (AGPT), IFA, immunoperoxidase assay (IPA), may be used directly. In general, these methods suffer from low sensitivity.

Detection of the IBV genome is sensitive and rapid (Jackwood et al 1992; Ramneek et al 2005) especially when used directly with infected tissue and has superceded many of the other assays. As, these techniques do not distinguish between infectious and non-infectious virus particles they are particularly useful for testing degraded diagnostic samples. Currently, the most common techniques utilize the RT-PCR and amplify a genomic region conserved across viral strains. After amplification, the PCR product may be characterized by sequencing and comparison with a database (Ramneek 2000; Lopez et al 2003) or restriction fragment length polymorphism analysis (Kwon et al 1993).

IBV infections can also be diagnosed by detecting the appearance or rise in titre of IBV-specific antibodies which occurs one week after infection (Ramneek 2000; Pei and Collisson 2005). Generally, for accuracy, paired samples are required. The first sample is taken at the onset of disease, and the second sample two to four weeks later (De Wit 2000). Alternatively, a specific IgM ELISA has been used to detect recent IBV infections (De Wit et al 1998) because of the limited lifespan of this antibody isotype. All IBV serotypes have both group and type-specific epitopes (the latter are present in the S1 protein (Cavanaght and Naqi 2003). In general the serological tests (ELISA, haemaglutination inhibition, immunofluorescence and immunodiffusion) bind to group, as well as to type-specific, antigens, so do not differentiate serotypes (Cavanaght and Naqi 2003).

Virus neutralisation is the reference test for the detection of type-specific antibodies (Cowan and Hitchner 1075). The specificity is very high after a single IBV inoculation (Karaca and Naqi 1993; De Wit et et al 1997), although it is not well standardised internationally, making it difficult to compare results between laboratories (De Wit 2000). Other disadvantage is that

the method requires reference strains and sera. That disadvantage can be avoided using monoclonal antibodies, but they are limited and do not cover the full range of IBV serotypes (Koch et al 1986).

2.4.9 Treatment

There is no specific treatment for IB. Some improvements in the management practices can help to decrease the impact of the virus: elimination of cold stress, overcrowding, and attempts to reduce live weight losses (e.g., increase the number of feeders). With nephropathic strains an additional supply of electrolytes can help in the balance of sodium and potassium (Cumming 1969).

2.5 Poultry immune system

The immune system in poultry, like that of humans, has developed a number of defence strategies to neutralise a wide range of pathogens. When pathogens enter the body they are repelled by the first line of defence, the innate immunity, such as physical and chemical barriers that prevent entry of the pathogen, and cellular and soluble components that are designed to eliminate the pathogen once it has gained entry (Erf 2004), such as phagocytic cells (Qureshi et al 2000), complement (Koppenheffer 1998), and natural killer cells (Sharma and Okazaki 1981).

Pathogens that cannot be denied access through physical barriers or controlled by the innate immune defence face a highly specific response (adaptive immunity), resulting not only in elimination of the pathogen but also as protection against repeat encounters (memory) with the same pathogen (Erf 2004). Adaptive immunity is mediated by a variety of cells. T-lymphocytes have helper or cytotoxic functions. The former help in the production of antibody by B cells and increase the microbicidal activity of macrophages and the latter are

involved in the lysis of virus-infected cells (Paul 2003). B cells are directly involved in humoral immunity and the production of antibodies against antigen (Sharma 2003). The immune cells reside in primary or secondary lymphoid organs. The thymus and Bursa of Fabricius increase in size until 1-2 months of age respectively and are the primary lymphoid organs. Secondary organs include the spleen, Harderian glands, bone marrow, conjunctival-associated lymphoid tissue, bronchial-associated lymphoid tissue and gut-associated lymphoid tissue. Chickens do not have lymph nodes but do have lymphoid nodules along the course of the lymphatics (Sharma 2003).

2.5.1 Immune response against IBV

2.5.1.1 Innate Immunity

In chickens, heterophils constitute the first line of defence against infectious agents and are the first cells to be recruited to the site of infection. However, it has been reported that heterophils have no effect on IBV multiplication (Fulton et al 1993). The role of the macrophage in IBV infections has not been totally determined yet, while no alterations in natural kill (NK) cell activity have been found post inoculation with IBV (Wakenell et al 1995). Pei et al (2001) reported that rChIFN-α inhibited replication of IBV (Beaudette strain) in chicken kidney cells (CKC), in a dose-dependent manner. Also, the supernatant from cultured spleen cells inhibited tracheal ring ciliostasis caused by IBV. Otsuki et al (1991) found no differences in IFN-γ levels of unstimulated lymphocytes from IBV-resistant and IBV-sensitive lines of chickens.

2.5.1.2 Humoral immunity

Chickens develop a humoral response to IBV infection, measurable by ELISA, HI or VN tests (Gough and Alexander 1977; De Wit et al 1992; Wilcox et al 1983). However, the precise

role of antibody in the control of IBV infections remains controversial. Several studies have shown that circulating antibody titres do not highly correlate with protection from IBV infection. Raggi and Lee (1965) reported a lack of correlation between infectivity, serological response and challenge with IBV vaccine. Gough and Alexander (1979) reported no correlation between HI antibody titres and susceptibility to challenge, as measured by re-isolation of virus from the trachea. Nevertheless, other studies demonstrated that humoral immunity plays an important role in disease recovery and virus clearance. Chandra (1988) showed that chickens treated with cyclophosphamide (B-cell ablation) had more clinical signs and more severe histopathological lesions in the kidney than untreated birds infected with IBV. Cook et al (1991), found that after inoculation with IBV, bursectomised chicks experienced a more severe and longer lasting infection than intact chicks, but mortality was unchanged. It has also been reported that high titres of humoral antibodies correlate well with the absence of virus re-isolation from kidneys and genital tract (Gough and Alexander 1977; MacDonald et al 1981; Yachida et al 1985) and protection against drop in egg production (Box et al 1988).

Following infection with a live IBV vaccinal strain there is a primary IgM response. As expected, the primary IgM response peaks, and declines, before that of the IgG response (Martins et al 1991). The secondary IgM response i.e. in response to a second challenge, peaks at the same time as that of IgG, but declines faster Anti-IBV IgG is detected in serum by ELISA at seven days post infection (p.i), reaching maximum levels at two weeks p.i and remaining positive until at least ten weeks p.i. (Pei and Collisson 2005). Dhinakar Raj and Jones (1996a) reported IgG antibody content was highest in tears on Day 7 post-infection (p.i.) and was still detectable on day 23 p.i. and that significant levels of IgG antibody were present in oviduct washes on days 7 and 23 p.i.

Holmes (1973) and Toro and Fernandez (1994) reported that local antibody (IgA) plays a role in protection of the respiratory tract, principally in the prevention of re-infections. On the other hand, Gelb et al (1998) found that some chickens with high tear IBV antibody titres were susceptible to IBV and that some chickens with low tear titres were protected. Dhinakar Raj and Jones (1996a) reported that tears showed the highest IgA antibody concentration on day 7 p.i. but this decreased to an insignificant level by day 17 p.i..

Pei and Collisson (2005) reported that antibody secreting cells (ASC) from the spleen and peripheral blood, reached their peak between seven and ten days p.i., correlating with the maximum virus load in the lungs, suggesting that ASC are not protective. These ASC (plasma cells) survived without antigen stimulation for at least 18 weeks p.i. Memory B cells in peripheral blood and spleen are generated from 3 to 10 weeks p.i and are important for protection against IBV infection (Pei and Collisson 2005).

2.5.1.3 Cellular immunity

Dhinakar Raj and Jones (1997b), using a T cell compromised chicken model, reported that T cell immunity is important in limiting the severity and lethality of the infections with IBV rather than clearing virus (measured by virus isolation). Studies reported by Seo and Collisson (1997), Seo et al (2000) and Collisson et al (2000) have shown a cytotoxic T lymphocyte (CTL) response that correlates with recovery from infection after an IBV infection. The CTL response is detected as early as day 3 p.i. with (22.9% lysis), peaks at 10 day p.i. (82.8% lysis) and falls to 46%, 38% and 23% on days 15, 20, and 30 p.i respectively (Seo and Collisson 1997). The majority of the CTL activity is dependent on the presence of $CD8^+$ cells, but a minority of effector cells may have a $CD4^+$ phenotype (Collisson et al 2000). Seo et al (2000) made an adoptive transfer of effector T cells, and showed that $CD4^-/CD8^+$ T cells bearing αβ T cell receptors, collected at 10 days p.i. with IBV, controlled acute IBV infection

in naïve chicks. Pei et al (2003) found that IBV-specific $CD8^+$ memory T cells generated at 3 to 6 weeks p.i. can protect sygeneic chicks from acute IBV infection.

In summary, the CTL response of chicks to IBV infection plays a critical role in impeding the injurious effects of virus during acute infection. Later control of infection depends on antibody, especially IgG, which is likely to play a major role in preventing recrudescence of infection

2.5.1.4 Passive immunity

Maternal-derived antibody (MDA) can be transferred via the yolk sac to neonatal chicks. This antibody (largely IgG) can interfere with the immunity generated by live vaccines of the same strain (Klieve and Cumming 1988). MDA produced by vaccination of breeders can provide protection of their chicks against IBV challenge until one week of age (Mockett et al 1987), but may not prevent viral infection of the respiratory system. Chicks hatched with high levels of maternal antibody had excellent protection (>95%) against IBV challenge at day 1 of age, but not at 7 days (<30%) (Mondal and Naqi 2001). However, Yachida et al (1981) showed that MDA did not reduce the titre of re-isolation of challenge virus given at two days of age.

2.5.2 Vaccination against IBV

2.5.2.1 Types of vaccines

Live, inactivated and DNA vaccines are available for IB immunization. Live vaccines are usually attenuated by serial passage in chicken embryos. Protection in the respiratory tract afforded by a single live vaccine has been reported by Gough and Alexander (1979) to be as short as six to nine weeks. These vaccines are commonly used in broilers (rarely in New Zealand) and as a priming vaccine in layers and breeders. Live vaccines can be administered

by eye drop, intratracheally or intranasally, as a coarse spray aerosol and in the drinking water.

Inactivated vaccines were developed with the objective of giving long-lasting immunity to hens and to protect against drops in egg production. However, single applications of inactivated virus vaccine induced little or no protection against egg loss (McDougall 1969; Box et al 1980; Muneer et al 1987) and no protection against loss of ciliary activity in the trachea (Martins et al 1991). These vaccines usually are given after 'priming' with the live virus and are administered a few weeks before egg production commences (Cavanagh and Naqi 2003). More recently, vaccines have been produced using DNA recombinant techniques. Song et al (1998) reported that a rS1 glycoprotein expressed by a recombinant baculovirus can induce a protective immune response as well as an antibody response. Kapczynski et al (2003) developed a DNA vaccination from the S1 gene, that when given either *in ovo* or intramuscularly, provided some protection against clinical disease after homologous IBV challenge. Tarpey et al (2006) reported promising results with the use of an experimental recombinant *in ovo* vaccine, based on the Beaudette strain which expressed a S1 protein from either Beaudette (Beau-R) or virulent M41 strains (BeauR-M41).

2.5.2.2 Vaccination programs

Different strains of IBV have been used as vaccines. The Massachusetts serotypes are used widely around the world because initial isolates from many countries were of that serotype (Cavanagh and Naqi 2003). Cross-protection tends to diminish as the degree of amino acid identity between the S1 proteins of two IBV strains decreases (Cavanaght el al 1997). The use of Ma5 vaccine (Massachusetts serotype) at 1 day old and the heterologous 4/91 vaccine at 2 weeks of age, was shown to be highly effective in protecting the respiratory tract of specific-pathogen-free chickens from many serotypes, isolated from disease outbreaks in different parts

of the world (Cook et al 1999b). That is the use of IB vaccines containing two or more antigenic types of IBV broadens the protection against challenge with IB isolates of many different serotypes.

In the New Zealand poultry industry a live vaccine (strain A) and an inactivated vaccine (Massachusetts type) are available. Findon (1987), reported that the live New Zealand strain A virus (49th egg passage), protected layers from IBV infection on farms with good management techniques but vaccination on another commercial farm gave less than ideal protection, due to intercurrent disease. Ramneek (2000) found that live A vaccine gave only partial protection in commercial broilers following experimental challenge with isolates C and D, as measured by lessening of pulmonary and renal histological lesions and viral excretion..

Timing of vaccination varies due to the titre of maternal antibody in chicks, vaccination methods used, and the needs or aims for control of IB, as well as other flock diseases. Broilers are commonly vaccinated by spray with live mild strain vaccines such as Massachusetts, H120 or Connecticut in the hatchery. Antibodies decline over time and, therefore, it is sometimes necessary to re-stimulate the immune system with subsequent live vaccinations (at 10 or so days of age), especially if the field challenge is high (Cavanagh and Naqi 2003). A typical vaccination programme (America, Europe) for parents and commercial layers involves three to four live vaccinations followed by a killed vaccination with a 3-week break between live vaccinations and a 6-week spread between the last live and killed vaccine. In farms with high IBV challenge, live boosting may be considered in the laying facility every 6-8 weeks in addition to the administration of a killed vaccine. In New Zealand, because of the mildness of the disease some layers may receive two live vaccines in rearing or alternatively one live and one killed vaccine. Broilers are rarely vaccinated.

Summary

IB is an important endemic disease of the poultry industry of New Zealand. IB shows winter seasonality but the cause of that is not clear. Some poultry meat consumers, and animal welfare lobbyists, in New Zealand (as in many other developed countries) are asking for the return to more ‘natural’ husbandry systems such as the rearing of free range chickens. Under this production system the birds may face many stressors such as extreme ambient temperatures that can put in jeopardy their homeostasis and so affect their welfare. The effect of the temperature on the immunity against endemic pathogens is not clear and studies show inconsistent results. Corticosterone is believed to be the principal glucocorticoid released by the birds in order to moderate the defence reactions to stress but its role in mediating the effects of temperature change on immune function is not clear.

Chapter 3

THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE PREVALENCE OF INFECTIOUS BRONCHITIS

3.1 Introduction

Seasonality of infectious diseases has been observed to exist since at least 400 BC, when Hippocrates stated 'whoever wishes to investigate medicine properly should proceed thus: in the first place to consider the seasons of the year' (Hare 1975).

Because seasonal cycles of infectious diseases are so universal and no single theory has proved satisfactory, explanations about their cause abound. More than one explanation or combination of explanations may be true and include: pathogen appearance, disappearance or changes in the genome, and environmental or host changes (Dowell 2001). Many diseases caused by the coronavirus such as severe acute respiratory syndrome (SARS) (Dowell and Ho 2004) and infectious bronchitis (IB) virus (Dhinakar Raj and Jones 1997b), exhibit winter seasonality with the presence of persistent virus carriers . A high flock prevalence of IBV has been demonstrated recently in New Zealand using RT-PCR, from samples collected during the winter-spring period (Ramneek et al 2005).

The peaking of respiratory diseases during winter on poultry farms is likely to be due to environmental factors such as ineffective ventilation because of the need to conserve heat. Reduced ventilation usually results in an increase in air pollutants, such as ammonia, carbon dioxide, dust and air-borne microorganisms (Anderson et al 1966). Certain factors are known to exacerbate clinical diseases or affect the immune responses: low/high environmental temperatures (Ratanasethakul and Cumming 1983; Henken et al 1983; Regnier and Kelly 1981), high ammonia levels (Anderson et al 1964; Kling and Quarles 1974), low levels of oxygen (Olander et al 1967), inspirable dust (Willis et al 1987), deprivation of foraging

material (El-Lethey et al 2003), and high levels of circulating corticosterone levels (El-Lethey et al 2003). Very few of these factors have been studied in relation to IBV infection with the exception of cold temperature (Ratanasethakul and Cumming 1983) and atmospheric ammonia (Kling and Quarles 1974). The accurate estimation of risk factors associated with IB disease events is critical to the determination of the benefit–risk ratio and the most cost-effective use of preventive therapies (Tonkin et al 2003).

The aim of this study was to firstly, determine the prevalence of IBV in broilers within the Canterbury province of New Zealand in late winter and spring and secondly, to determine whether a particular management or environmental factor was related to the presence of IBV.

3.2 Materials and methods

3.2.1 Farms

The farms eligible for the case control study were suppliers for a major broiler producer in Canterbury, New Zealand. Ten farms (the average size was 77,000 birds), with birds between 3 to 4 weeks of age, were selected from a total of 29 farms that produced approximately 60-70% of the broiler chickens of Canterbury. The chicks arrived at the farms from a hatchery where a live and dead vaccination programe was carried out in the breeders (chicks had maternal antibody titres of approximately 3000 (ELISA TropBio Pty Ltd, Queensland, Australia), which declined to negative levels by 3 weeks of age. The broiler chicks themselves were not vaccinated against IBV.

Birds from one of the sheds of each farm had some production (reduced live-weight gain) or respiratory signs (lacrimation, sneezing, coughing within the last 5 days) that suggested the presence of IB. Such a shed was defined as a case shed, and a matched control shed was selected as a shed containing birds that were believed not to have been affected by IB. That is, each farm had a case and a control shed within which flocks had a high degree of similarity

with respect to the age of birds, number of drinkers, nature of food and litter (wood shavings). All sheds were a controlled environment. An attempt was made to minimize contamination between sheds by personnel adhering to certain hygiene activities: changing of overalls, disinfection of boots. The most common ventilation system was 'cross ventilation', where air is drawn into the shed due to negative pressure by fans, circulated across the width of the shed and out of the shed through vents on the opposite wall. Fans ran for approximately 30 sec every 5 minutes at three days of age, which increased over the next three weeks to 50% of the time (running for 2.5min in every 5min).

The stocking density of the birds in the farms was 21 birds per square metre. Shed separation varied from farm to farm but there was a minimum distance of 10 metres between them. Lighting patterns varied from farm to farm; the most common system was 4 hours on-2 hours off. The birds ate standard grower pellets with a protein level of 21%.

3.2.2 Clinical signs and detection of IBV

In each shed, 6 birds were randomly selected from the clinically unaffected population.
In flocks unvaccinated to IBV within a region of high IBV prevalence (a previous NZ study by Lohr (1974), showed 69 % IBV seroprevalence), the number of birds needed to be sampled in order to give 80% test power with 95% confidence was 6 (Cannon and Roe 1982).

Birds were checked individually for tracheal rales (a sound emanating from the bronchi, also detected by vibrations when holding a chick), nasal and ocular discharge.
The trachea of each of the 6 live birds was swabbed by inserting a cotton swab into the trachea and gently swabbing the wall. Additionally, cloacal swabs from the same birds were taken by inserting a cotton swab deeply into the vent followed by rotation against the mucosal wall. The tracheal or cloacal swabs were pooled together (each shed) and then placed in 20 ml

of transport media (TM) at 4 °C (Poulvac Sterile diluent, Fort Dodge Animal Health, USA) containing antibiotic (Enrofloxacin 10%, Bayer, New Zealand). The samples were centrifuged in the laboratory at 3000g 4 °C for 10 minutes, then 200 µl of the fluid plus 500 µl TRizol (Life Technologies, USA) and 100 µl of Chloroform (Biochemical, England) were vortexed for 10 seconds. This was followed by an incubation period of 10 minutes at room temperature and centrifugation at 13000 rpm for 15 minutes at 4 °C. The upper colourless phase was collected in separate tubes and mixed with 250 µl isopropanol (Analar R, Biochemical, England) , followed by incubation at room temperature for 10 minutes. Again the samples were centrifuged at 13000 rpm for 15 minutes at 4 °C. The pellet was added to 400 µl of 70 % Ethanol (Biochemical, England), vortexed for 3 seconds and centrifuged (13000 rpm). The supernatant was discarded and the pellet was air dried in a sterile environment. Finally the pellet was dissolve in 20 µl of diethylpolycarbonate-treated water and stored at – 20 °C.

RT-PCR reactions were carried out using the 'Titan One Tube RT-PCR System' (Roche Diagnostics GmbH, German) described by Ramneek (2000) and Lopez et al (2006). In the RT-PCR reaction, the primers NT1 and NT2 (Ramneek 2000) were used to bind to the N-terminus of the S1 gene (+116-308) and bracketed a hypervariable region (HVR1) (Cavanagh et al 1988). The RT reaction was carried out at 45 °C for 30 minutes in a Gen Amp PCR system 2400 PCR machine (Applied Biosystems, USA), followed by a pre-PCR denaturation at 94 °C for 20 seconds, denaturation at 94 °C for 20 seconds, annealing at 50 °C for 15 sec, polymerisation at 72 °C for 40 seconds for the first 10 cycles, and a incremental 5 seconds for each of the next 25 polymerisation cycles. An additional polymerisation step followed at 72 °C for 7 minutes. The PCR product was visualised on a 1 % agarose gel in Tris borate EDTA buffer containing ethidium bromide (0.5 µg/ml), using an ultraviolet transilluminator (UVP Lifesciences, UK).

3.2.3 Ambient measurements

The levels of ammonia, oxygen, carbon dioxide, and relative humidity, were measured daily (9 am in the morning after a feeding period). Duplicate measurements were taken, 0.5 m above the litter surface in the the middle of the shed using a Multiwarn II gas detector (Dräger AG, Lübeck, German). Litter humidity was measured by manual compression in a scale of 1 to 4 (A score of 1 being dry litter with moisture around 20% and 3 is the highest level considered acceptable for broiler production – an in house company score). The mean outdoor temperature for the period of time when the measurements were made (August) was 10.3 °C and the monthly rainfall was 86 mm (NIWA Science, National Climate Centre, NZ).

3.2.4 Questionnaire and data analysis

A questionnaire was completed by the farm manager in order to provide information about certain farm characteristics such as: flock size, management practices and environmental factors (see appendix I). A descriptive analysis was completed to provide summary statistics for all variables in the data set. Binary logistic regression analyses were conducted to assess associations between the presence of IBV and various risk factors, using MINITAB Statistical Software (Minitab Inc, Pennsylvania, USA).

3.3 Results

IBV was detected by RT-PCR in 50% of the farms visited (Figure 3.1), from 5 of the 10 case sheds and 1 of the 10 control sheds. The virus was found in either the trachea or the cloaca (1 farm trachea, 2 farms cloaca), or in both (2 farms) (appendix II). In only 2 of the 5 positive farms where IBV was detected was there accompanying clinical respiratory signs suggestive of IB (lacrimation, sneezing, coughing). Ambient humidity was the only risk factor studied

that showed a significant inverse relationship with the prevalence of IBV (odds ratio with 95% confidence levels less than, and excluding, 1 are significant and depict a negative or inverse association) (Table 3.1).

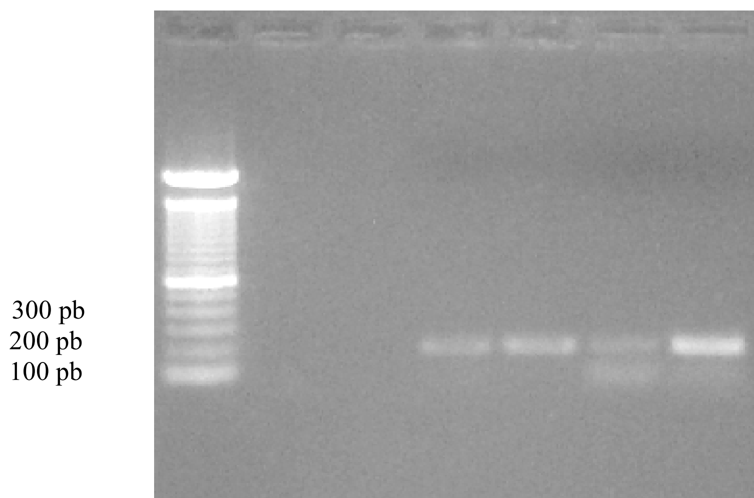


Figure 3.1. RT-PCR amplimers using the primers NT1 and NT2. Lane 1. MW marker, Lane 2. Blank reation, Line 3. Negative control, Lines 4-7. Samples containing IBV

Table 3.1. Logistic regression analysis of factors associated with the presence of IBV in broiler farms.
OR= Odds Ratio, CI= Confidence interval, SEM= Standard error of the mean
*Measurement taken 0.5 m above litter surface

Risk Factor	P Value	OR	95% CI	Mean (Range)	SEM
Temperature*	0.99	1	0.74-1.35	22.5C (20-29°C)	0.720
Relative humidity*	0.05	0.92	0.84-1	75.3% (50-97%)	3.490
Ammonia*	0.4	1.03	0.96-1.1	16.7 ppm (0-50 ppm)	3.100
Oxygen*	0.25	0.07	0.0-7.4	20.4 % (20.0-20.9 %)	0.054
Carbon dioxide*	0.8	0.69	0.01-60.39	0.31% (0.0-0.8%)	0.040
Litter humidity	0.1	4.6	0.74-28.47	2.6 (1.0-3.5)	0.220

3.4 Discussion

There is a belief that IBV infection in broilers in New Zealand is under-diagnosed, particularly as its prevalence has been largely based on seroconversion and this may be misleading as birds are frequently tested at slaughter (35-40 days old) and have had little time to seroconvert, thus leading to false negatives. A study conducted by Lohr (1974) showed that 69% of the New Zealand broilers and 75% of layers were sero-positive to IBV, as detected by neutralising and precipitating antibodies. Ramneek et al (2005) showed the farm prevalence (predominantly broilers) of IBV to be 19%, using RT-PCR. Ignjatovic and Sapats (2000) reported that IBV is prevalent in all countries with an intensive poultry industry, with the incidence of infection approaching 100% in many locations. Shapouri et al (2004) reported a prevalence of 42.8% in Iran (strain 4/91), Gutierrez-Ruiz et al (2000) reported a prevalence in Mexico of 56.5% and in Italy Capua et al (1994) showed a prevalence of IBV of 81.8% (strain 624/I). In the present study, IBV was detected by RT-PCR in birds from 5 of the 10 case sheds and 1 of the 10 control sheds. In 2 of the 5 case sheds where IBV was detected there were accompanying clinical signs suggestive of IB. Infectious bronchitis may resemble other respiratory diseases such as Newcastle disease, laryngotracheitis and infectious coryza. Newcastle disease has not been reported as a clinical entity in New Zealand (Durham et al 1980), but an avirulent lentogenic strain is occasionally detected in commercial poultry flocks (Howell 1992). Larnygotracheitis shows more marked respiratory signs than IB (Cavanagh and Naqi 2003) and is uncommon in broilers in New Zealand (Lohr and Saywell 1976; Poland 2006). Infectious coryza can be differentiated on the basis of facial swelling that rarely occurs in IB and was not present in these broilers.The lack of clinical disease in the field where IBV is present could be attributed to a number of factors, including: mildness of the strains found (IBV), good management practices and the absence of major immunosuppressive agents such

as infection bursal disease virus – eliminated from New Zealand since 2000 (Chai et al 2001), chicken anaemia virus – controlled by vaccination of breeders (Stanislawek and Howell 1994; Markowski-Grimsrud and Schat 2003) and inter-current infections such as; *Mycoplasma gallisepticum* (MG) – breeders tested negative and *E. coli* . In addition, Ramneek (2000), studied the pathogenicity of 5 different genotypes of IBV and found that all the strains analysed induced only mild histological lesions and clinical signs when poultry were housed under good management conditions, with respect to temperature, relative humidity and levels of ammonia.

Managers of all of the flocks followed a common operation manual established by the broiler producer, in order to follow best husbandry practice. The narrow range of values within the environmental variables (temperatures etc) studied meant that is was difficult to distinguish between particular management practices among farms and IBV prevalence (appendix I).

Environmental changes are the factors most often provided to explain the seasonality of infectious diseases (Dowell 2001) and most avian respiratory pathogens exhibit an annual increase in incidence each winter. In this study, only 2 of 10 farms exceeded the optimal temperature for birds of 19°C to 24°C as defined by Yoder et al (1977) and one of these farms had birds positive for IBV. A reduction in ambient temperature (constant 16°C) has been reported to increase mortality with IBV infections (Cumming 1969) even though there were no indications (postural adjustments, behavioural changes, or changes in food and water consumption) that the birds were uncomfortable with the low ambient temperature. None of the farms in this study had temperatures below 20°C.

In this trial we found a significant inverse relationship ($p = 0.05$; Odds Ratio (95% OR) = 0.84-1.0) between the prevalence of IBV and relative humidity (Table 1). Yoder et al (1977) also reported that at medium temperatures (19-24°C) and low humidity, flocks infected with

Mycoplasma synoviae and IBV had a higher incidence of airsaculitis than at a high humidity. It has been proposed that drying of mucosal surfaces increases the probability of transmembrane spread of bacteria (Dowell 2001). In humans, outbreaks of respiratory syncytial virus (RSV) have been reported to peak in seasons of lower relative humidity (Chew et al 1998). In addition, Ijaz et al (1985) studied the survival of airborne human coronavirus and reported that a high relative humidity (80 +/- 5%) at 20 +/- 1°C, was found to be least favourable for the survival of virus aerosols (half-life about 3 h) compared with low humidity (50 +/- 5%) where the half-life of the virus was 67 +/- 8 h.

In this trial, there was no significant relationship between litter humidity and IBV occurrence. Indeed in the majority of the of the sheds positive for IBV, levels of litter humidity were higher than a recommended level of approximately 25% (North and Bell 1990). The level of moisture in poultry litter has been positively associated with other diseases such as coccidiosis (Waldenstedt et al 2001), poult enteritis and mortality syndrome (Edens et al 1998) and salmonellosis (Carr et al 1995).

Based on the results of previous studies (Anderson et al 1964; Kling and Quarles, 1974) it was anticipated that higher levels of ammonia could increase the presence of IBV or exacerbate the clinical signs found in infected birds. Kling and Quarles (1974) found that the birds housed under high levels of ammonia had significantly smaller Bursae of Fabricius than the controls. Anderson et al (1964) reported that 72 hours of exposure to ammonia concentrations in the range of 20 to 50 ppm significantly increased the infection rate of chickens with Newcastle disease virus when given as an aerosol. However, no association was found between IBV prevalence and ammonia concentration, in the present study. Atmospheric levels of oxygen and carbon dioxide in the poultry industry are used principally as criteria for an efficient ventilation system. Although 55% of the sheds in this study had low

levels of oxygen (under 20.5 %) and 40% had high levels of carbon dioxide (over 0.3 %), there was no significant association between the levels of oxygen or carbon dioxide and susceptibility to IBV.

It can be concluded, within the narrow constraints of the management systems described, that humidity had an influence on the presence of IBV, but there was no influence due to temperature, ammonia, carbon dioxide or oxygen levels, or litter humidity.

Chapter 4

EFFECT OF THERMAL CHANGE ON IMMUNE RESPONSES AND PHYSIOLOGICAL FUNCTION IN CHICKENS

4.1 Introduction

The poultry industry continues to experiment with 'natural' husbandry systems such as the rearing of free range chickens. Under such farming systems birds that have been primarily selected on the basis of rapid growth in optimal management systems encounter a variety of pathogens and, at the same time, experience various kinds of physical, climatic and social stresses (Van Loon et al 2004). Chickens subjected to high or low temperatures exhibit several physiological and behavioural changes that allow them to re-establish thermal balance with their surroundings (Cooper and Washburn 1998). Chickens housed at high temperatures decreased feed consumption in order to reduce metabolic heat production and maintain homeothermy, leading to lower growth rates. At high temperatures, decreases in blood haematocrit values have been reported (Hutson 1965; Deaton et al 1969), although this is not always consistent (Altan et al 2000). Conversely, Deaton et al (1969) showed that birds reared at low temperatures (7.2^{o}C) had significantly higher haematocrit levels than birds reared at 23.9 or 32°C.

One important cause of mortality in modern broiler operations is ascites. In an international ascites survey of 18 countries in four continents, ascites affected 4.7% of live broilers (Maxwell and Robertson 1997). Cold environmental temperatures have been reported as an important factor that triggers the onset of ascites or the pulmonary hypertension syndrome (PHS) in birds (Julian 2005).

A number of studies have indicated that birds under stress due to housing conditions (El-Lethey et al 2003) or temperature change (Altan et al 2003) display protracted tonic

immobility (TI) and have higher than normal levels of corticosterone in the blood. Both parameters have been used as indicators of welfare status (Duncan et al 1986; Feltenstein et al 2003). Although Zulkifli et al (1999) reported that high temperatures did not affect the TI interval in chickens, Hangalapura et al (2004b) showed that exposure to low temperatures resulted in lower corticosterone levels in the plasma of chickens.

In wild birds, energy-demanding processes such as breeding, brood care and migration may interfere with the immune response and reduce health status, especially during cold seasons (Lochmiller and Deerenberg 2000). Thermoregulation is a significant energy-demanding process that may compromise the immune system (Hangalapura et al 2003). Nonetheless, results from studies on the effects of low or high environmental temperatures on humoral and cell-mediated immune responses in chickens are inconsistent. Although Donker et al (1990) found no effect of temperature on antibody production, Heller et al (1979) reported immune enhancement in birds exposed to short periods of high temperatures. In contrast, Subba Rao and Glick (1970) and Mashaly et al (2004) described immunosuppression in chickens exposed to high temperatures for a longer period. Equally contradictory, short term exposure at low temperatures has been reported to enhance antibody production (Subba Rao and Glick 1977) and *in vitro* lymphocyte proliferation (Hangalapura et al 2003) or, conversely, to have a negative impact on humoral (Svensson et al 1998) and cell mediated immunity (Regnier and Kelly 1981) in birds. The variability between results can be attributed, in part, to the specific immunologic parameters measured, assay techniques and the different breeds of chickens used.

Infectious bronchitis (IB) is a highly contagious endemic disease of chickens characterized by respiratory and renal pathology (Cavanagh and Naqi 1997). It is caused by the infectious bronchitis virus (IBV) which was first described in New Zealand by Pohl (1967). Under

optimal environmental conditions IBV NZ strains induce only mild histological lesions and clinical signs (McCausland et al 1972; Ramnnek 2000), but cold conditions have been associated with more severe pathology in Australia (Ratanasethakul and Cumming 1983). There is a perception in the New Zealand poultry industry that certain environmental stressors, principally temperature change, precipitate the onset of respiratory problems characteristic of IB.

In this experiment environmental temperatures were changed to affect the biological function and adaptive capacity of chickens following infection with an endemic pathogen as compared with previous studies (El-Lethay et al 2003; Mashaly et al 2004; Hangalapura et al 2004a), where immunological responses to novel antigens were assessed (e.g., keyhole limpet haemocyanin; tetanus toxoid, human serum albumin). The 'affective states' of the animal (Fraser 2004) were assessed by measuring levels of corticosterone in plasma, and tonic immobility, and compared to various estimates of immunocompetency.

4.2 Materials and methods

4.2.1 Animals and housing conditions

One hundred day-old broiler chickens (Cobb breed) were obtained from a commercial hatchery. The birds were placed in cages and provided with *ad libitum* access to a commercial broiler ration and drinking water until 42 days of age. Following hatch the birds had steadily decreasing ambient light (23h $\rightarrow$ 16h per day) and temperature (30°C $\rightarrow$ 20°C) over three weeks. At 20 days of age the birds were weighed and randomly assigned to one of three temperature treatments (n = 30), either high (30 +/- 2°C), or moderate (20 +/- 2°C) or low (10 +/- 2°C), with an ambient relative humidity of 45-55% (Figure 4.1). The rooms (one isolation room for each treatment) were heated individually and monitored by a computerised thermosensor.

Experimental procedures were conducted in accordance with the requirements of the Lincoln University Animal Ethics Committee.

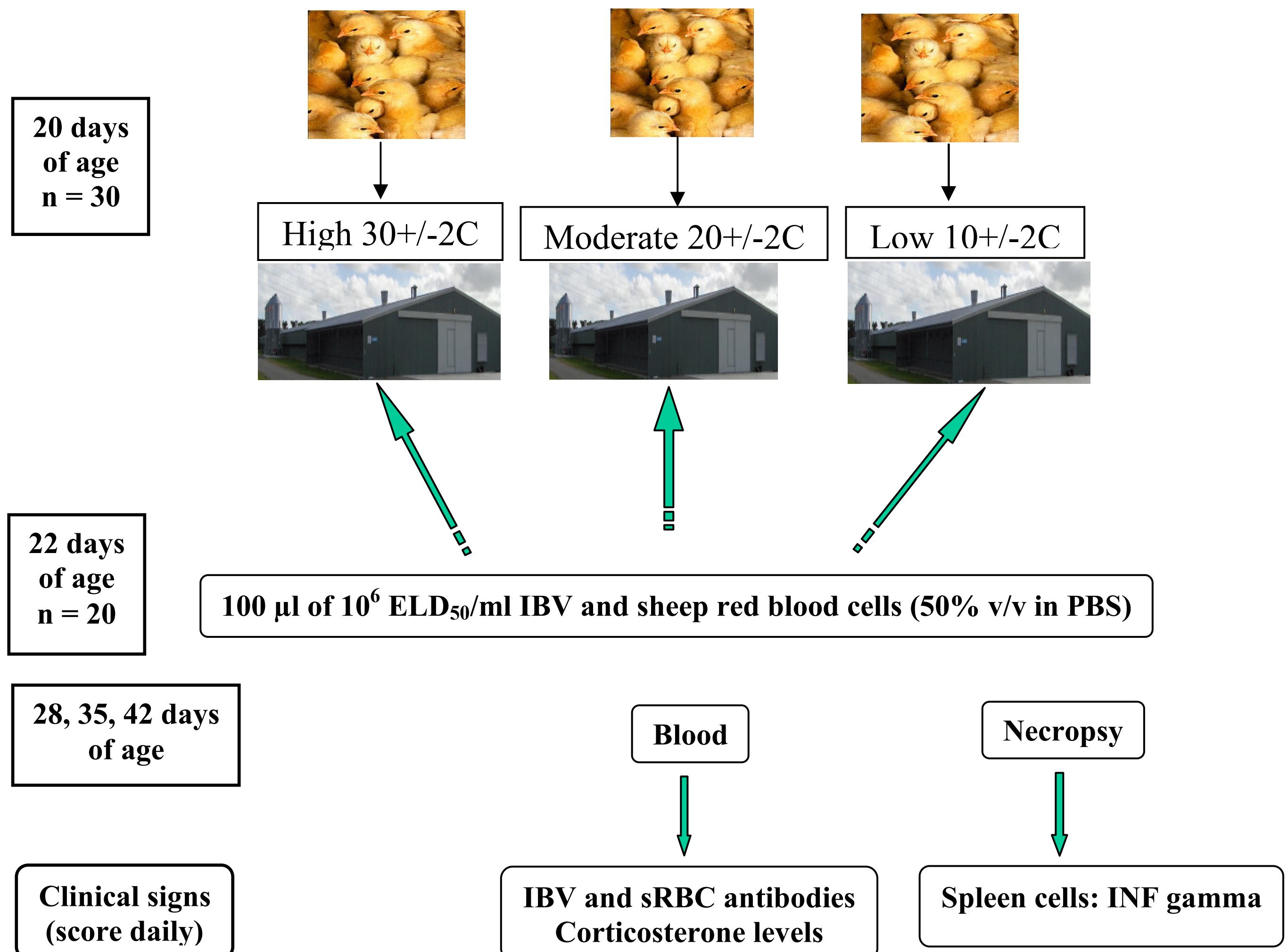


Figure 4.1 Experimental design to test the effect of thermal change on immune response and physiological function in chickens

4.2.2 Immunisation

Pure IBV (NZ strain C) stocks (Ramneek et al 2005) were amplified and titred in IBV-free, 9-day-old chicken embryos. These were used for challenge or for the production of ELISA antigen. As there was no access to SPF eggs at the time of the experiment (from Australia or New Zealand) and given that the transport of IgG from the yolk to the embryonic circulation

is minimal until day 15 (<100µg/ml) (Kowalczyk et al 1985) it was decided to use commercial 9-day-old chicken embryos. Virus was inoculated into five embryos/dilution (10^{-1} to 10^{-8}) and the eggs were incubated for 7 days at 37 °C, candling daily for mortality. On the seventh day all the remaining eggs were opened and examined for evidence of IBV lesions in the embryos (curling, stunting, etc). The Reed and Muench (1938) calculation was used to calculate the EID_{50}. From the 30 birds in each temperature tested group, 20 of similar body weight were challenged with IBV and subsequently studied. When virus antigen was needed for the ELISA, allantoic fluid was centrifuged at 48,000 *g* for three hours, heat- inactivated at 56°C for 20 minutes (Cavanagh and Naqi 1997) and the total protein concentration was determined by the Bio Rad D_C protein assay (Porex Bio Products Group, USA).

Different concentrations of the mitogen concanavalin (Con A) and purified IBV proteins were tested for their ability to activate splenocytes from 4-weeks-old chickens previously challenged with IBV (Figure 4.2; Figure 4.3). Con A was used a positive control in the splenocyte activation assays.

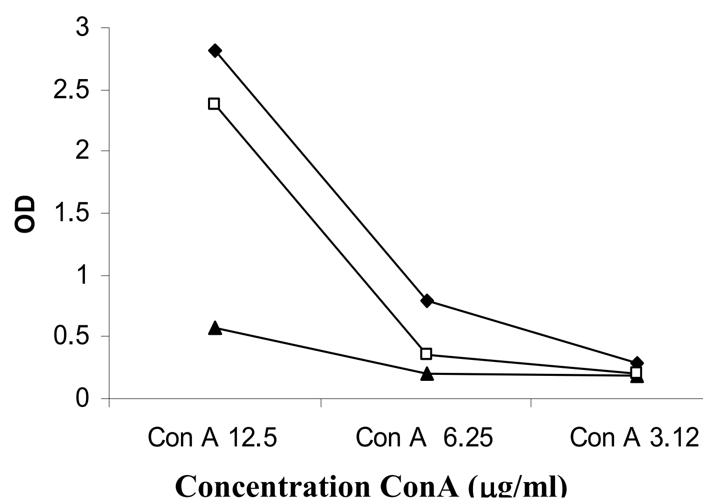
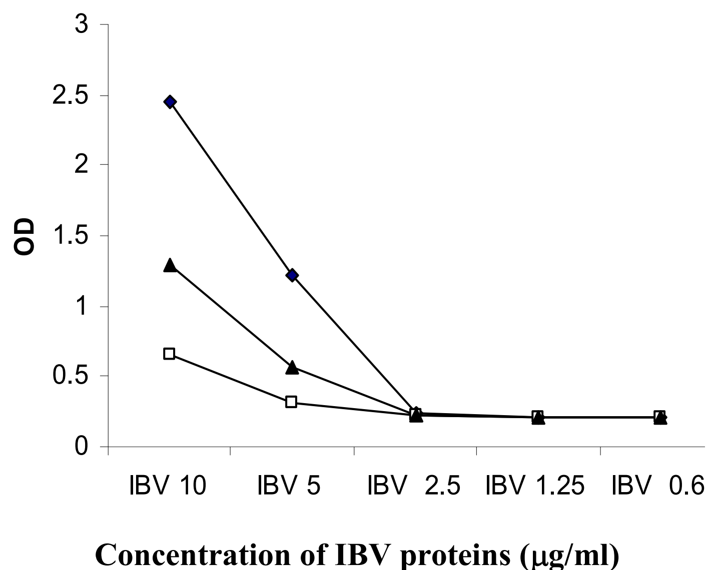


Figure 4.2. Mitogenic response of chicken splenocytes to Con A with varying concentrations of cells: 1 x 10^7 cells/ml (♦), 5 x 10^6 cells/ml (□), and 2.5 x 10^6 cells/ml (▲)



Concentration of IBV proteins (µg/ml)

Figure 4.3. Relationship between recall antigen (µg/ml), and cell concentration: 1 x 10^7 cells/ml (♦), 5 x 10^6 cells/ml (□), and 2.5 x 10^6 cells/ml (▲)

At 22 days of age, all birds were infected intranasally with 100 µl of 10^6 EID_{50}/ml of IBV. At the same time, 0.5 ml of packed sheep red blood cells (50% v/v in PBS) was injected into the right breast muscle of all birds.

4.2.3 Blood sampling

Eight one-day-old chickens were slaughtered to measure maternally-derived serum antibody levels against IBV (ELISA). At 14, 20, 22, 28, 35 and 42 days of age, blood samples (1.5 ml) were taken from the brachial vein of 6 birds per treatment group within three minutes of capture using a hypodermic syringe. Following clotting samples were centrifuged at 2,300 rpm for 10 minutes, the serum was aspirated and stored at -70°C. At 42 days of age blood was taken to determine the haematocrit.

4.2.4 Clinical signs

Respiratory signs were visually scored (number of birds showing that particular sign) three times a day, following the method described by Avellaneda et al (1994), with some modifications (scores of 0-3 instead of 1-4, to facilitate analysis between treatment groups):

0 = no clinical signs; 1 = lacrimation, slight head movement, sneezing, coughing; 2 = lacrimation, tracheal rales, nasal exudate, depression; 3 = more marked signs plus swollen heads. These scores were pooled within groups, and the final score was the mean of the scores on that day (Appendix III).

4.2.5 Lesions and mortality

The number of dead birds was recorded daily. Gross lesions in both the dead and sacrificed birds (from each treatment group n = 6; on days 20, 22, 28, 35 and 42) were evaluated.

Air-sacs were evaluated, a modification of Kleven et al 1972, using a scale of 0 to 3: 0 = no lesion; 1 = slight cloudiness moderate exudate; 2 = thickened with accumulations of cheesy exudate confined to a single air sac; 3 = as in 2, but lesions found in two or more air sacs.

Trachea: 0 = no lesions; 1 = serous or catarrhal exudate; 2 = hyperaemia and caseous exudate; 3 = haemorrhage and/or caseous exudate.

Liver as described by Avellaneda et al (1994) but with a scale of 0 to 3: 0 = no lesions; 1 = slight inflammatory process; 2 = severe inflammation and or formation of pseudomembrane; 3 = presence of pseudomembrane or haemorrhage.

Heart as described by Balog et al (2003): 0 = no lesions; 1 = mild heart enlargement; 2 = distinct, severe heart enlargement with minimal pericardial fluid; 3= severe right ventricular hypertrophy, copious fluid with or without fibrin clots.

Kidney: 0 = no lesions; 1 = swollen; 2 = swollen and pale; 3 =swollen and pale plus ureters distended with urates.

Composite lesion scores were calculated by multiplying the number of birds having a particular score and adding them together for each time point and treatment group.

4.2.6 Body weights, haematocrit and body temperature

Body temperatures were obtained with a thermometer inserted approximately 3 cm into the cloaca for 30 seconds (Cooper and Washburn 1998; Altan et al 2003). Temperatures were taken at the same time (am) of the day to minimize diurnal fluctuations. The birds were weighed after euthanasia. The haematocrit was determined using heparinised capillary tubes followed by centrifugation in a micro-haematocrit centrifuge (Haemofuge A, Heraeus Sepatech, Germany) for 7 minutes (Altan et al 2000).

4.2.7 Tonic immobility (TI)

Tonic immobility was induced by placing birds in a U-shaped plastic cradle covered with a cloth and restraining them for 15 seconds, as per Jones and Faure (1981). The time, after the 15 initial seconds of restraint, was recorded until the birds made the righting response. Failure to obtain a TI reaction lasting at least 10 seconds resulted in exclusion of the bird from analysis. For welfare reasons the test was terminated if the birds had a righting response greater than 15 minutes and a maximum score of 900 seconds was assigned (El-Lethey et al 2003).

4.2.8 Corticosterone

The corticosterone levels were measured by radioimmunoassay (RIA) at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, N.Z, as described by Littin and Cockrem (2001).

4.2.9 Antibody

Following clotting and storage at 4°C, the blood was centrifuged at 1500 rpm for 15 mins and serum aspirated and stored at -70°C. Antibody titres to IBV were determined by Tegel Foods

Ltd, New Plymouth, using a commercially available ELISA kit (TropBio Pty Ltd, Queensland, Australia), containing the IBV Vic S strain as capture antigen. Antibody production against SRBC was measured using an haemagglutination test (1:2 dilution), as described by Wegmann and Smithies (1966).

4.2.10 Interferon gamma

The cell mediated immune response was assessed by the levels of interferon gamma produced by splenic lymphocytes cultured *in vitro* (Lambrecht et al 2000). During necropsy the spleen was aseptically removed and crushed manually into RPMI 1640 medium and the resultant spleen cells filtered through gauze. Splenocytes were separated on Ficoll-Paque™ Plus (Amersham Biosciences, Sweden), washed twice in RPMI 1640 medium (Sigma-Aldrich, USA), and resuspended in the same medium supplemented with 10% FCS. The recall antigen was derived from a virus stock of IBV strain C, produced in 10-day-old embryonated eggs. One hundred µl of the recall antigen (IBV) at a concentration of 9µg/ml was added to the 96 well plate containing 100 µl of the cell suspension at a concentration of 1 x 10^7/ml, in triplicate, and was incubated at 39°C for 72 hours. An equivalent number of purified splenocytes were cultured with Con A at 12.5µg/ml, as a positive control. Supernatants were collected after centrifugation at 3000rpm for 15 minutes and stored at -70 °C.
Levels of IFNγ in the culture supernatants were estimated using a sandwich ELISA (Biosource International, USA). Plates (Nunc Maxisorp, catalog # 468667) were coated (100 µL/well) with chicken anti-IFNγ monoclonal antibody (catalog # 5C12308, Biosource, USA) at a concentration of 2 µg/mL for 18hours at 2-8 °C. The coating antibody was aspirated from the wells and tapped on absorbent paper to remove excess liquid. The plates were then blocked by adding 300 µL of blocking solution to each well (Appendix VI), for 2 hours at room temperature. After 3 washes, the supernatants from the splenocyte cultures were added (100 µL/well) followed immediately by the addition of 50 µL of biotinylated antibody and

incubated for 2 hours at room temperature with continual shaking (700 rpm). After 3 washes, 100 µL/well of the chromogen TMB (catalog # SB01, Biosource, USA) was added and incubated for 30 minutes at room temperature with continual shaking (700 rpm). Then, 100 µL/well of Stop Solution (catalog # SS01, Biosource, USA) was added. The optical density of the plates was measured in a microfluorometer (BMG, Germany) at 450 nm, within 30 minutes of adding the Stop Solution.

4.2.11 Statistical analysis

MINITAB Statistical Software (Minitab Inc, USA) was used for calculating the confidence intervals for body weights, haematocrit, body temperature, antibody levels, interferon gamma production, tonic immobility and plasma corticosterone levels. A two-way table was constructed in order to analyze the independence of the clinical signs using Chi square. The Fisher's exact test was used to analyse the lesion results (since Chi square was not valid for some of the tables).

4.3 Results

4.3.1 Clinical signs

The numbers of chickens showing clinical signs of IB, that is having a clinical score of 1 or more, differed significantly (by Chi square analysis) between the low and both moderate and high temperature groups, from day 3 after IBV challenge ($p=0.001$), until day 12 ($p=0.030$) peaking at day 4 ($p=0.000$) post challenge (Figure 4.4).

The birds under high or low temperature exhibited unusual physical positions. At high temperature in addition to panting, the birds were standing with the neck extended and wings held away from the body. At low temperature the feathers of the birds were fluffed out and the birds were crowded together (Figure 4.5).

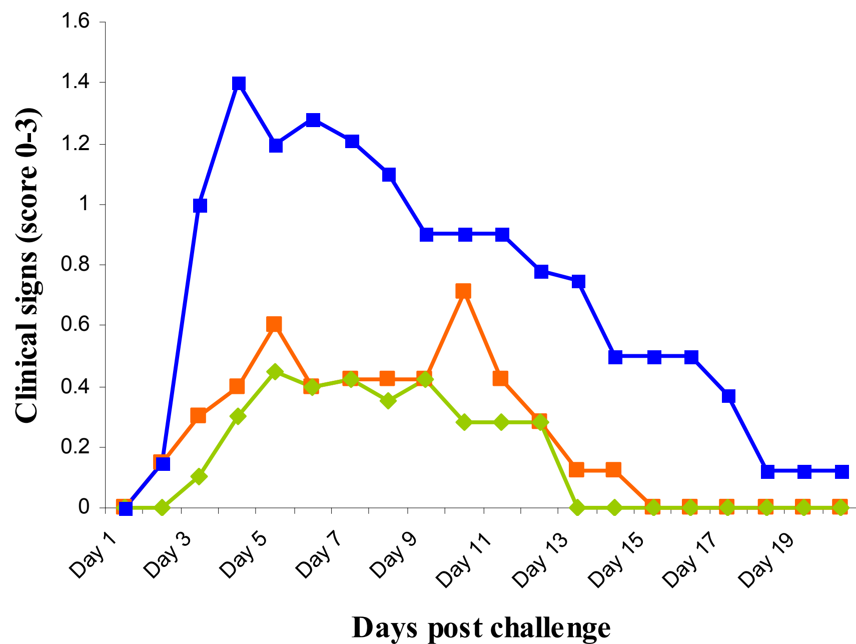


Figure 4.4. Mean clinical score (0-3) of birds housed under high (■), moderate (●), or low temperature (■)

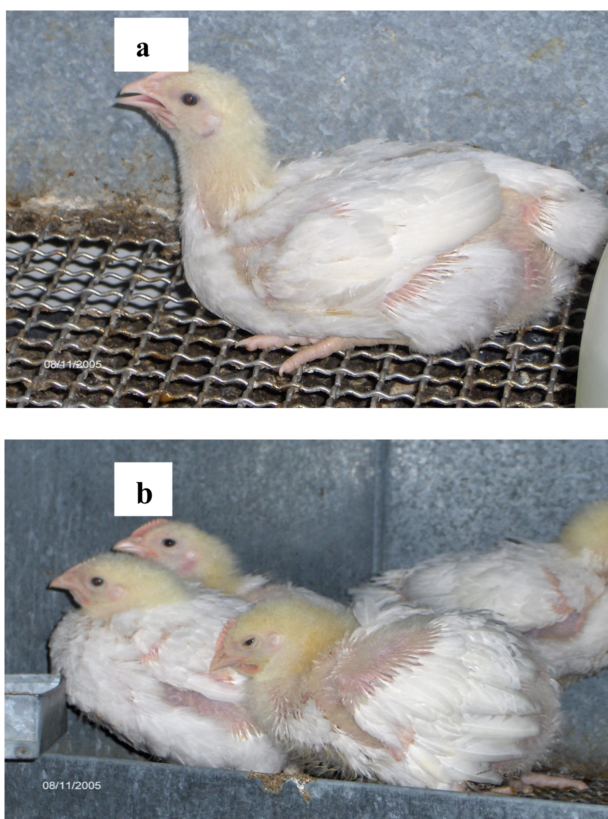


Figure 4.5. (a) Bird under high temperature with its neck extended, (b) birds under low temperature crowded together

4.3.2 Lesions and mortality

The numbers of birds with air sac lesions housed under high and low temperature (combined) were higher than those of birds under moderate temperature, 6 days after IBV challenge (p=0.043: Fisher's exact test). (Figure 4.6) (Appendix IV)

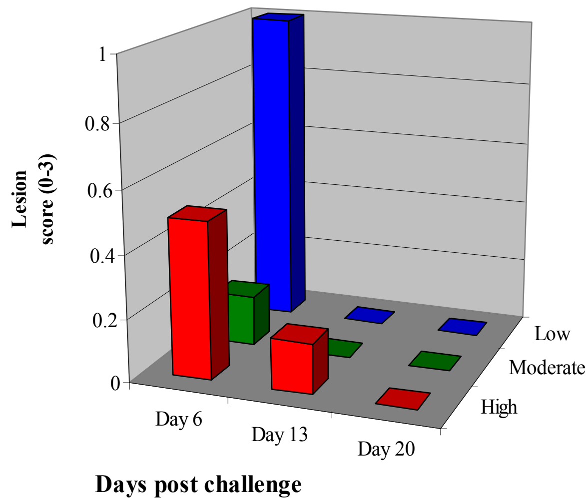


Figure 4.6. Mean air sac lesions score of birds housed at different temperatures

The numbers of chickens with liver lesions at 13 days after challenge was greater in the extreme (high or low) temperature rooms than in the moderate temperature room (p=0.013: Fisher's exact test) (Figure 4.7; 4.8) (Appendix IV)

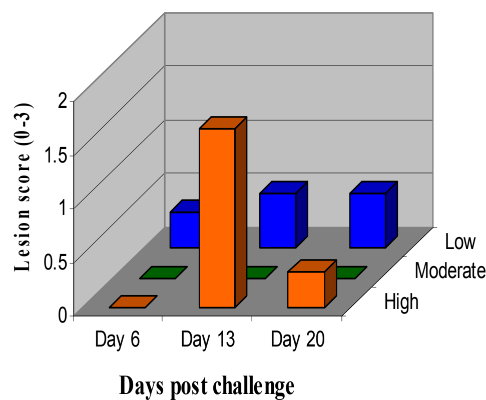


Figure 4.7. Mean lesion (liver) score of birds housed under different temperatures

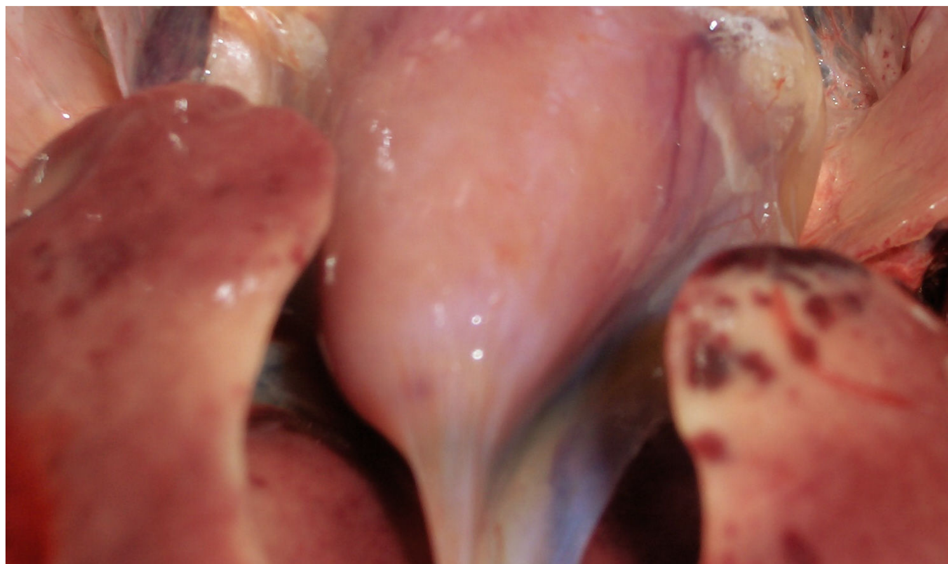


Figure 4.8. Liver haemorrhages found in a naturally dying bird after being housed under high temperature for 10 days

At day 13 after challenge, the number of chickens with heart lesions (hydropericardium, ventricular hypertrophy) (Figure 4.9) was greater in the low than in the high temperature group (p=0.015: Fisher's exact test). The cumulative mortality, by the end of the trial (20 days after challenge) in the birds housed under low moderate and high temperatures was 5% (1 of 20) 0% and 10% (2 of 20) respectively.

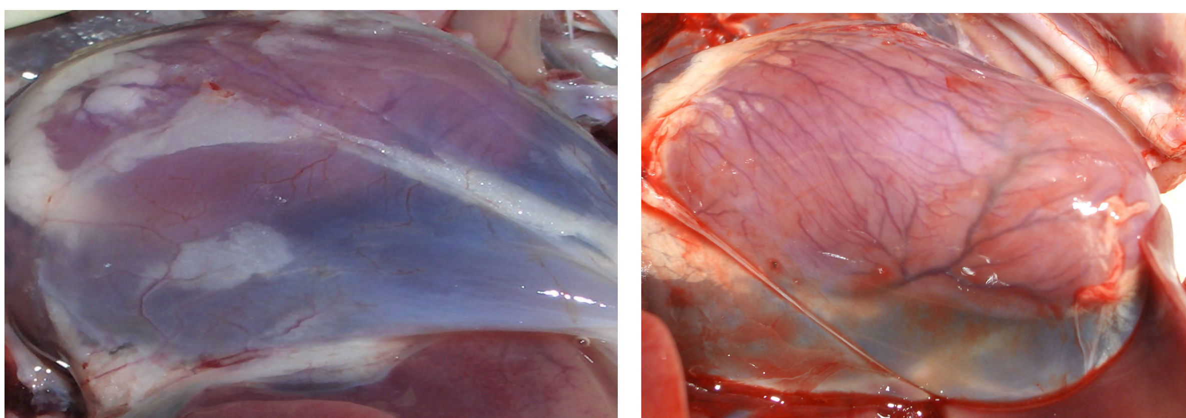


Figure 4.9. Hydropericardium in a bird dying after being housed under low temperature for two weeks

4.3.3 Weight gain, haematocrit and body temperature

The chickens housed under high temperature grew significantly slower ($p<0.05$) than the birds under moderate temperatures. The haematocrit levels at the end of the trial (42 days of age; 20 days after IBV challenge) were lower ($p<0.05$) in the birds housed at high temperatures compared with those housed at low or moderate temperatures (Table 4.1).

Table 4.1. Confidence intervals for mean body weight, haematocrit and body temperature of birds housed under high (H), moderate (M) or low (L) temperatures

Variable	Temp	Mean grams	SE Mean	95% Confidence Interval	
				Lower	Upper
Body weight at 42 days of age	H	1.792	0.012	1.76	1.82
	M	2.032	0.039	1.93	2.13
	L	1.693	0.113	1.40	1.98
Haematocrit at 42 days of age	H	29.83	0.40	28.8	30.9
	M	33	0.816	30.9	35.1
	L	34.83	0.401	33.8	35.9
Body temperature at 42 days of age	H	42.5	0.289	41.8	43.2
	M	42	0.129	41.7	42.3
	L	42	0.258	41.3	42.7

4.3.4 Tonic immobility (TI) and plasma corticosterone (CORT) levels

At 21 days (1 day after the initiation of temperature treatment, but prior to IBV challenge) and 41 days of age the birds housed under low and high temperatures had higher TI indices ($p<0.05$) than those housed under moderate temperature (Figure 4.10).

At 28 days of age (8 days after the beginning of the temperature treatment), the group of birds exposed to low temperatures had a significantly higher mean plasma corticosterone concentration ($p<0.05$) than birds housed under moderate temperatures (Figure 4.11) (Table 4.2).

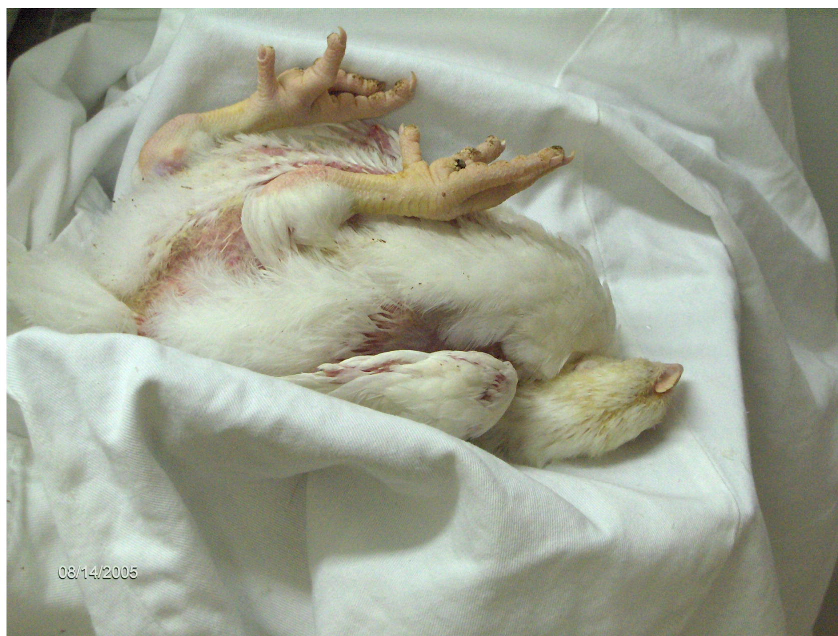


Figure 4.10. Bird in a U-shaped plastic cradle during the TI test

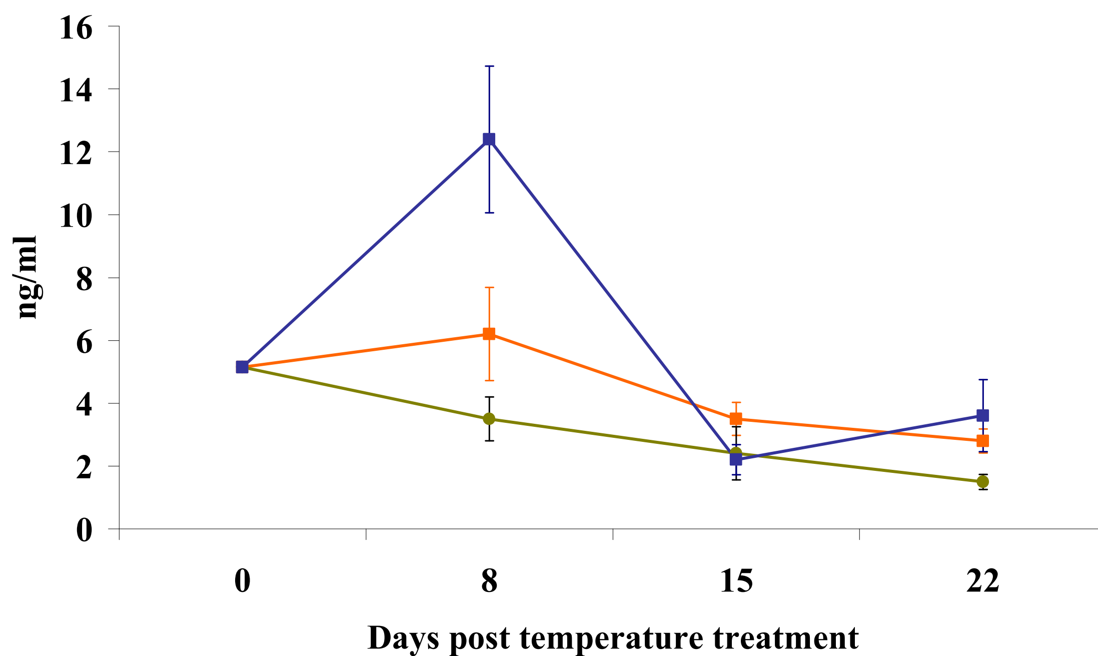


Figure 4.11. Mean plasma corticosterone concentrations of birds housed under high (■), moderate (●), or low temperature (■)

Table 4.2. Confidence intervals for mean plasma levels of corticosterone concentration (CORT levels) of birds housed under high (H), moderate (M) or low (L) temperatures

Variable	Temp	Mean	SE Mean	95% Confidence Interval	
				Lower	Upper
CORT levels day 6 post challenge	H	6.2	1.48	2.34	9.96
	M	3.5	0.697	1.54	5.41
	L	12.4	2.33	6.38	18.36
CORT levels day 13 post challenge	H	3.5	0.52	2.19	4.88
	M	2.4	0.85	0.23	4.59
	L	2.2	0.48	1	3.46
CORT levels day 20 post challenge	H	2.8	0.38	1.84	3.80
	M	1.5	0.24	0.86	2.11
	L	3.6	1.15	0.67	6.59

4.3.5 Humoral immune response

At one day of age the birds had high titres of maternally-derived antibodies against IBV, which progressively declined to zero by 22 days of age when IBV was administered intranasally and SRBC was injected (Figure 4.12). By thirteen days after IBV challenge (35 days of age), the mean antibody titre was lower in birds housed under high ($p<0.05$) compared with those under moderate temperatures and there was a tendency for lower antibody levels in birds housed under low temperatures (Table 4.3).

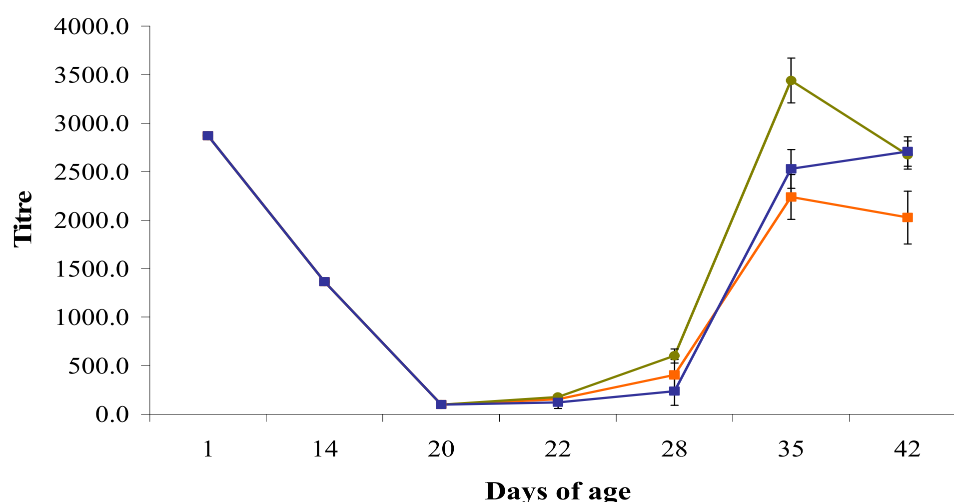


Figure 4.12. Antibody titre to IBV measured by ELISA from chickens housed under high (■), moderate (●), or low temperature (■)

By six days after SRBC injection (28 days of age) the chickens housed at high temperature had a significantly higher antibody titre (end point) against SRBC than the birds under low or moderate temperature (Figure 4.13) (Table 4.3).

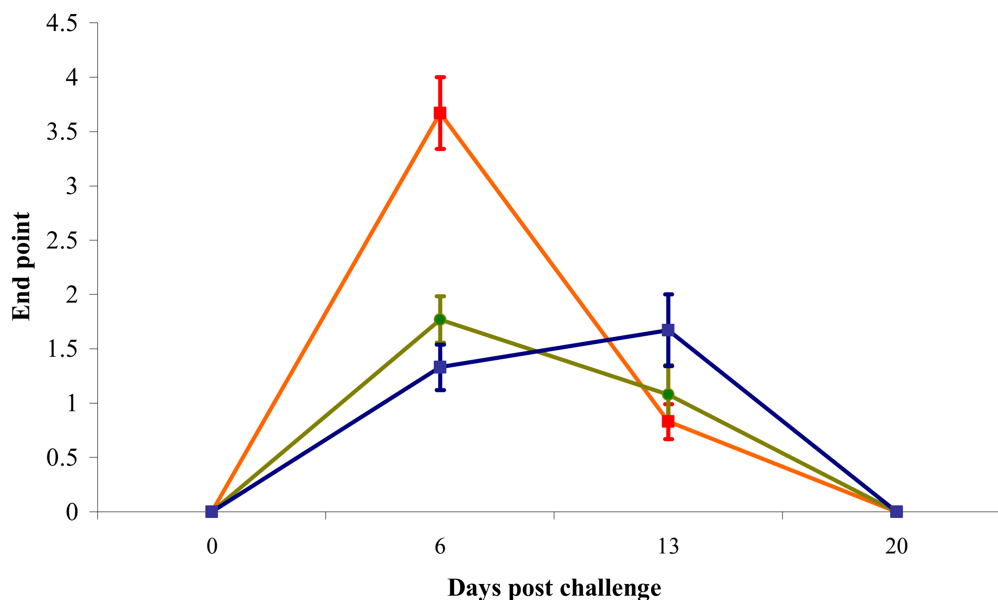


Figure 4.13. Haemagglutinin titre to SRBC of birds housed under high (■), moderate (●), or low temperature (■)

Table 4.3. Confidence intervals for IBV antibodies and haemagglutinin titres of birds housed under high (H), moderate (M) or low (L) temperatures

Variable	Temp	Mean	SE Mean	95% Confidence Interval	
				Lower	Upper
Antibodies IBV, day 13 post challenge (35 days of age)	H	2238	230	1647	2829
	M	3440	200	2926	3954
	L	2528	230	1937	3119
Antibodies IBV, day 20 post challenge (42 days of age)	H	2027	274	1323	2731
	M	2673	152	2282	3064
	L	2708	145	2335	3081
Haemaglutination (SRBC), day 6 post challenge (28 days of age)	H	3.67	0.333	2.81	4.25
	M	1.67	0.211	1.12	2.21
	L	1.33	0.211	0.79	1.88
Haemaglutination (SRBC), day 13 post challenge (35 days of age)	H	0.83	0.167	0.4	1.26
	M	1.08	0.271	0.39	1.78
	L	1.67	0.33	0.81	2.25

4.3.6 Cell mediated immune response

The levels of interferon gamma released by splenic lymphocytes after *in vitro* stimulation with IBV antigen tended to be lower in the high and low temperature groups 13 days after challenge, but the difference was not statistically significant. By twenty days post challenge the levels of interferon gamma were significantly greater ($p<0.05$) in the birds housed under high temperature than in those housed under cold temperature (Figure 4.14) (Table 4.4).

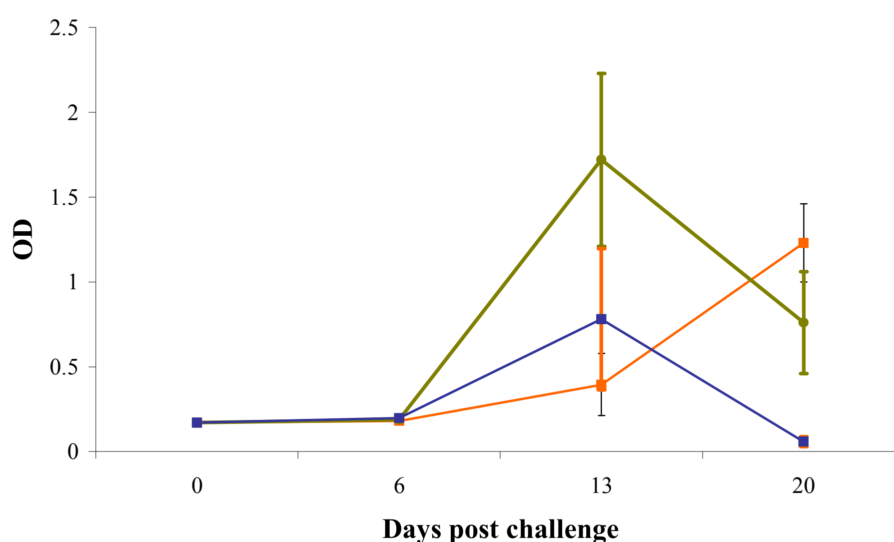


Figure 4.14. Levels of interferon gamma released from spleen cells stimulated with IBV antigen and measured by ELISA, from chickens housed under high (■), moderate (●), or low temperature (■)

Table 4.4. Confidence intervals for INF gamma levels of birds housed under high (H), moderate (M) or low (L) temperature regimens

Variable	Temp	Mean	SE Mean	95% Confidence Interval	
				Lower	Upper
INF-Gamma, day 13 post challenge (35 age)	H	0.39	0.183	-0.08	0.86
	M	1.73	0.506	0.42	3.03
	L	0.79	0.417	-0.29	1.86
INF-Gamma, day 20 post challenge (42 age)	H	1.23	0.234	0.63	1.83
	M	0.77	0.299	0	1.54
	L	0.06	0.0291	-0.02	0.13

4.4. Discussion

The effects of temperature on the manifestation of disease following challenge with IBV (NZ, strain C) have been demonstrated. It was not possible in this trial to distinguish between pathology due to temperature differences *per se* and the effects of temperature plus IBV infection. However, given the high prevalence of IBV within poultry units of New Zealand (Lohr, 1974, Ramneek et al, 2005), the effect of environmental temperature on infected birds is important to note.

When broilers were housed under a moderate environmental temperature, clinical signs were apparent 2-3 days after infection, peaked at 5-7 days and disappeared within 10-14 days in agreement with other studies (Hofstad 1984; Dhinakar Raj and Jones 1997; Ramneek 2000). Low and high temperatures exacerbated the respiratory signs and lesions in birds infected with IBV.

The increase in mortality of birds subjected to low temperatures resulted from lung oedema, probably secondary to the pulmonary hypertension syndrome (Cumming 1969; Julian 2005). It has been reported that low housing temperatures produce changes of osmolarity in the mucous layer on bronchial walls that can elicit respiratory alterations or pathologies such as bronchospasm (Gani et al 2003). Furthermore, several birds in the low temperature group developed ascites, obvious upon necropsy. However, Tottori et al (1997) reported that IBV may also induce ascites in broilers. In this experiment severe air sac lesions were not found and this may reflect the mildness of the strain of IBV (Ramneek 2000) or the lack of co-infections such as *Mycoplasma synoviae* in the breeding stock (Hopkins and Yoder 1982).

A high proportion of the birds housed under high temperatures showed hepatic lesions, principally enlarged pale liver with haemorrhages. This is likely to be due to endotoxic shock due to high temperatures, as described by Crespo and Shivaprasad (2003) and Julian (2005).

The longer duration of the clinical signs and higher mortality in the birds housed at high or low temperatures, in comparison with birds under moderate temperature, could be attributed to lower production of IBV antibody, 2 weeks after infection. Humoral immunity is an important protective mechanism in the later stages of IB, as shown by Cook et al (1991), where bursectomised chicks experienced a more severe and longer lasting infection than intact chicks.

In agreement with previous reports (Vo et al 1978; Cooper and Washburn 1998), the chickens housed in high temperature showed significantly decreased growth; at the end of the experiment (day 42), the birds weighed less than those housed at moderate temperatures and there was a trend for the low temperature birds to have lower body weights. One interpretation of this is that birds spend more energy on thermoregulation and, thus, have less available for body growth. It has also been reported that chronic heat exposure significantly decreases protein digestion (Larbier et al 1993). As feed intakes were not measured, differences in food conversion efficiencies due to temperature were unknown.

The high temperatures were associated with a reduction in packed cell volume (PCV) (29% versus 33% in the moderate temperature group), which was similar to results reported by Luger et al (2001) and likely to be associated with haemodilution, an adaptive response enabling water loss by evaporation without compromising plasma volume, with most of the evaporative water loss coming from the extracellular compartment (Darre and Harrison 1987; Altan et al 2000). The tendency for an increased PCV in birds housed in the cold temperatures could be caused by a decline in plasma volume as a result of plasma leakage from the blood vessels as part of the development of ascites or, less likely, by an increase in erythropoiesis as a compensatory reaction to oxygen lack in the tissues or to the high levels of corticosterone (Luger et al 2003).

The specific antibody response to IBV was measured using an indirect ELISA method with the VicS IBV strain as capture antigen - previously shown to crossreact with all NZ strains (Ramneek 2000). The antibody response (presumably a mixture of IgM and IgG and predominantly the latter) was significantly reduced in birds housed under high temperatures, in agreement with Zulkifli et al (2000) and Mashaly et al (2004), who showed that heat-stress caused a reduction in antibody synthesis *in vivo* to Newcastle Disease Virus and SRBC. Wang et al (2001) reported that acute heat stress decreased the release of T-helper 2 cytokines in rats, which are important for antibody production (Lebman and Coffman 1998). Mujahid et al (2005) reported that heat stress in chickens stimulates free oxygen radical production, which is known to have several non-specific damaging effects on the immune system. Perhaps, the requirement for nutrients to maintain an efficient immune response was not met in the current experiment due to a reduction in feed intake (not measured in this trial) plus low feed digestibility (proteins, fat, starch), that occurs in birds under heat stress (Bonnet et al 1997).

In the present study, low temperatures tended to reduce IBV titres in peripheral blood but this was not significant. Hangalapura et al (2004a) reported that cold stress (10°C, a similar temperature to that used in the present experiment) did not affect specific antibody response to keyhole limpet haemocyanin (KLH). However, Hester et al (1996) reported that a short exposure to 0°C decreased antibody response of single caged hens but not of hens in colony cages, presumably indicating the thermal advantage of collective huddling. Parmentier et al (2002) and Svensson et al (1998) reported a low energy requirement for the humoral immune response in poultry. In the present experiment there was an unlimited supply of feed and so the birds in low temperatures may have met the energetic requirements of immunity and growth by increasing food intake to avoid any trade-off between these demands and demands for other needs such as growth.

As compared to the above results, the birds housed at high temperatures developed significantly higher levels of haemaglutinating antibodies to SRBC than birds housed under low or moderated temperatures, 6 days after injection (28 days of age). This response was similar to that observed by Heller et al (1979) who reported that chickens (Rhode Island Red x Leghorn) housed at 41.5+/- 1°C had an enhanced humoral response to SRBC when there was a time-lapse of 24 to 96 hours between immunisation and heat exposure (a time-lapse of less than 24 hours did not have any effect). Furthermore, Regnier et al (1980) reported that New Hampshire birds, housed for 5 days at 36°C and inoculated intravenously with SRBC 24 hours before thermal treatments, had higher titres to SRBC 10 days after vaccination. Similarly, with cold stress of chickens there was an increase in immune response, measured as increased IgM antibody production and a markedly reduced the IgG antibody titre (Subba Rao and Glick 1977).

It has been reported that mild stress or relatively brief stress responses can enhance certain immune responses including innate (Brines et al 1996), humoral (Grayson et al 1981; Cupps et al 1985), and cell mediated immunity (Wiegers et al 1993). However, heat stress has also been reported to cause immunosuppression in commercial layers, housed at (35°C for 5 weeks, Mashalay et al 2004) or to have no immunological effect at all (36°C for 5 days; Regnier et al 1980). The differences in the humoral responses to IBV and SRBC may be explained by the fact that the haemagglutination test principally detects the levels of IgM in response to a challenge with SRBC and the IBV ELISA used in this experiment measures both the levels of IgM and IgG (but predominantly the latter), 6 weeks after infection.

As in mammalian systems, studies in poultry have shown that the measurement of chicken interferon gamma released by T cells after *in vitro* stimulation gives a good evaluation of CMI (Prowse and Pallister 1989; Lambrecht et al 2004), which is a major protective

mechanism against IBV (Seo and Collisson 1997). Cell mediated immunity was not significantly affected by heat stress or cold stress in the first 13 days of treatment with a tendency to be less but at 20 days the levels of interferon gamma in the birds subjected to low temperatures were lower than in the high temperature group. These results extend those of Mashaly et al (2004) who showed that non-specificT cell activities (mitogen proliferation assay) of chickens one week after heat exposure were not significantly affected by heat stress, and Regnier and Kelly (1981) who also reported that low temperatures suppressed non-specific cell mediated immune responses (measured by phytohaemagglutinin skin test) after 5 days of exposure at 1°C. Hangalapura et al (2004a) reported that low temperatures can enhance cell mediated immune response (lymphocyte proliferation). In mice, Iwakabe et al (1998) reported that NK activity and IFN-γ production from cultured spleen cells was greatly reduced when the animals were under stress (physical restraint).

Interpretation of the difference in the immune response to high or low temperatures between different studies is complicated by the nature and duration of the stressor [10°C in the present experiment and with Hangalapura et al (2004a) versus 0°C in the trial by Hester et al (1996)], difference between lines of chickens [Cobb broilers in this trial versus commercial layers with Mashalay et al (2004)] and immunological parameters under investigation [IFN-γ production in the present experiment versus lymphocyte proliferation with Hangalapura et al (2004a)].

The birds housed in low and high temperature had a longer tonic immobility period, in accordance with previous findings from Altan et al (2003), but in contrast to other reports where no effect followed housing at 36°C for 6 hours (Zulkifli et al 1999).

It was found that only the birds housed under low temperatures had a peak of plasma corticosterone concentration (one week after the start of treatment) in agreement with Luger et al (2003) (broilers), but in conflict with a report by Hangalapura et al (2004b) who showed

that low temperature decreased plasma corticosterone levels in layer birds. In general, plasma corticosterone concentrations decreased after the peak (one week after treatment), which could reflect adaptive mechanisms (Post et al 2003) such as changes in the level of plasma corticosteroid binding protein (Turner 1986).

The immunosuppressive effects of stress have been hypothesed to be via the production of glucocorticoids despite the fact that some researchers have demonstrated that stress-induced suppression of immunity can be produced in an adrenal-independent manner, suggesting that a variety of other hormones, neurosecretory products and cytokines may be involved (Keller et al 1983; Bonneau et al 1993. In this trial levels of corticosterone were not associated with immune response; that is the corticosterone peak in the cold treatment group,1 week after challenge, was not associated with immunosuppression or enhancement.

In conclusion, the clinical signs and lesions produced by IBV were exacerbated under extremes of temperature. This is the first study to show the effect of temperature on the immune response to IBV in broilers. Cell mediated (INF γ) and humoral (antibody) immunity to IBV was affected by housing temperature. The increased tonic immobility period and levels of corticosterone in chickens were also linked to house temperature and indicate welfare concerns. There was no evidence linking the levels of corticosterone to immunosuppression in birds housed at high or low temperatures. The cost that the broilers have to pay in order to retain or regain homeostasis is important from both immunological and production points of view. More specific experiments are needed to better characterise the mechanisms that modulate immune function during stressful episodes.

Chapter 5

EFFECT OF CORTICOSTERONE ON THE IMMUNE RESPONSE IN BROILER CHICKENS

5.1 Introduction

From the time chicks are hatched and throughout the rest of their lives, different stressors confront them. The physiological regulation of homeostasis is achieved by complex endocrine interactions, principally by the hormones secreted from the adrenal gland (Harvey et al 1984). Different forms of stress are directly or indirectly translated to the body by two commonly used endocrine pathways (Post et al 2003). These two arms of the stress response consist of the sympathetic-adrenalmedullary axis, resulting in the release of catecholamines, and the limbic-hypothalamo-pituitary-adrenocortical axis, which causes the release of glucocorticoids. Several studies have pointed out that neuro-endocrine-immune interactions may affect the immune response (Savino and Dardenne 1995; Cohen et al 1999). It has been reported in several animal species that hormones secreted during a stress response can produce either stimulatory or inhibitory effects, depending on the nature and duration of the stressor and the immunological parameter under investigation (Sapolsky et al 2000; Pruett 2001).

Corticosterone (CORT) is the principal glucocorticoid released by adrenal glands in birds in response to hypothalamic-pituitary activity (Harvey et al 1984). Its physiological function is in restraining the defence reactions to stress, which would themselves become damaging if left uncontrolled (Munck et al 1984) and it redirects metabolism to meet the energy demands during stress (Ingle 1954). CORT released by the adrenal gland in response to a stressful situation is mostly transported bound to plasma proteins (Carsia and Harvey 2000), but the free (i.e. non-protein bound) moiety passes readily from the circulation through the plasma membrane of cells eventually to reach specific nuclear receptors namely the mineralocorticoid

receptors (MRs) and glucocorticoid receptors (GRs) (Carsia and Harvey 2000). Unliganded GRs and MRs exist as monomers in a multiprotein complex with several chaperones, such as heat-shock proteins and immunophilins (Bamberger et al 1996). The hormone-receptor complex translocates to the nucleus, and after forming homo or heterodimers or associating with other proteins (Bamberger et al 1996), initiates either the activation or suppression of specific genes (Boumpas et al 1993).

Most organs and physiological systems in birds have cells with receptors that are responsive to CORT including the brain (Koehler and Moscona 1975), thymus (Gould and Siegel 1984), Bursa of Fabricius (Sullivan and Wira 1979) and liver (Tu and Moudrianakis 1973). It has been reported that long exposure to CORT produces metabolic and behavioural changes in birds. These included decreases in growth and body weight (Hayashi et al 1994; El-Lethey et al 2001), atrophy of lymphoid organs (Dohms and Metz 1991), increased fat deposition, largely in the abdomen and liver (Saadoun et al 1987) and increased tonic immobility (TI) duration (Jones et al 1988; El-Lethey et al 2003).

The involvement of endogenous glucocorticoids in the host's defence against infection has been highlighted by studies with adrenalectomised animals. In those studies, increased mortality was found in adrenalectomised animals after injection with *E. Coli*, bacterial lipopolysaccharide, IL-1β, or TNF-α; this increased mortality was essentially eliminated by administering exogenous glucocorticoids to adrenalectomized animals prior to challenge (Bertini et al 1988; Gonzales et al 1993; Kapcala et al 1995)

Numerous studies (Griffin 1989; Sapolsky et al 2000; Isobe and Lillehoj, 1992, 1993) have shown that pharmacological doses of CORT can inhibit the production of many cytokines, e.g. IL-1, IL-2, IL-3, IL-5, IL-6, IL-8, IL-12, interferon γ (IFN-γ), tumour necrosis factor α (TNF-α), granulocyte–macrophage colony-stimulating factor (GM-CSF), by suppressing

transcription, translation, and secretion or by destabilizing its mRNA (Knudsen et al 1987; Wiegers and Reul 1998) or by inducing a decoy receptor that binds and sequesters without transmitting any activity (Colotta et al 1993). Surprisingly, glucocorticoids given at high doses *in vitro* have been shown to up-regulate the expression of various cytokine receptors, which is in apparent contrast to the other 'restraining' effects of these hormones: e.g. for IFN-γ (Strickland et al 1986), IL-2 (Fernandez-Ruiz et al 1989) and IL-6 (Campos et al 1993). Furthermore, it has been reported that eosinophils cultured *in vitro* express increased levels of major histocompatibility complex (MHC) class II molecules in the presence of glucocorticoids and either IL-3, IL-5 or GM-CSF, leading to enhanced antigen presentation (Guida et al 1984). This paradox (inhibition *vs* enhancement) is not well understood in terms of its molecular mechanism or physiological significance (Wiegers and Reul 1998). It has been assumed that the factors which alter the balance between inhibition and enhancement are the amount of corticosterone released and/or the duration of the stress situation (Davis et al 1997; Dhabhar 2002).

The goal of this study was to assess the effect of stress (as mimicked by the administration of physiological doses of CORT), on both humoral and cell-mediated immune responses in chickens challenged with infectious bronchitis virus (IBV), and injected with sheep red blood cells (SRBC) and phytohaemagglutinin (PHA).

5.2 Materials and methods

5.2.1 Titration of oral corticosterone treatment

In order to establish the appropriate quantity of CORT required to changen immune function and track the pharmacokinetics of CORT in 20 day-old commercial broilers, a pilot trial was carried out. Two different doses of CORT (Sigma Chemical Co, USA) were administered via the drinking water (15.4mg/l and 7.7mg/l) to two groups and a third untreated group was a

control (all, n=8). All the birds were injected in the breast muscle with SRBC [0.5 ml of packed sheep red blood cells (50% v/v in PBS)] two days after the start the treatment with CORT (day 22 of age). At 6 and 10 days post vaccination with SRBC serum was collected and the anti-SRBC antibody titre was assessed by haemagglutination test (1:2 dilution), as described by Wegmann and Smithies (1966).

5.2.2 Animals, housing conditions and corticosterone treatment

In the main trial, one hundred day-old broiler chickens (Cobb breed) were obtained from a commercial hatchery. The birds were housed under a controlled environment, placed in cages and fed *ad libitum* from the day of hatching until they were 42-day-old. They were subjected to decreasing light and temperature as outlined in Chapter 4.

At 22 days of age the birds were weighed and randomly divided in three equal groups (33 birds per group), housed at 20-21°C and provided with eight hours of continuous light until 42 days of age.

The first group was the control [not challenged with infectious bronchitis virus (IBV) and not treated with CORT], the second group was not exposed to exogenous CORT but was challenged with IBV. The third group was treated via the drinking water with CORT (15.4 mg/l; Sigma Chemical Co, USA) from day 22 until the day 42 of age and challenged with IBV (Figure 5.1). Treatment groups were segregated in separate isolation rooms with separate air sources and sanitary procedures.

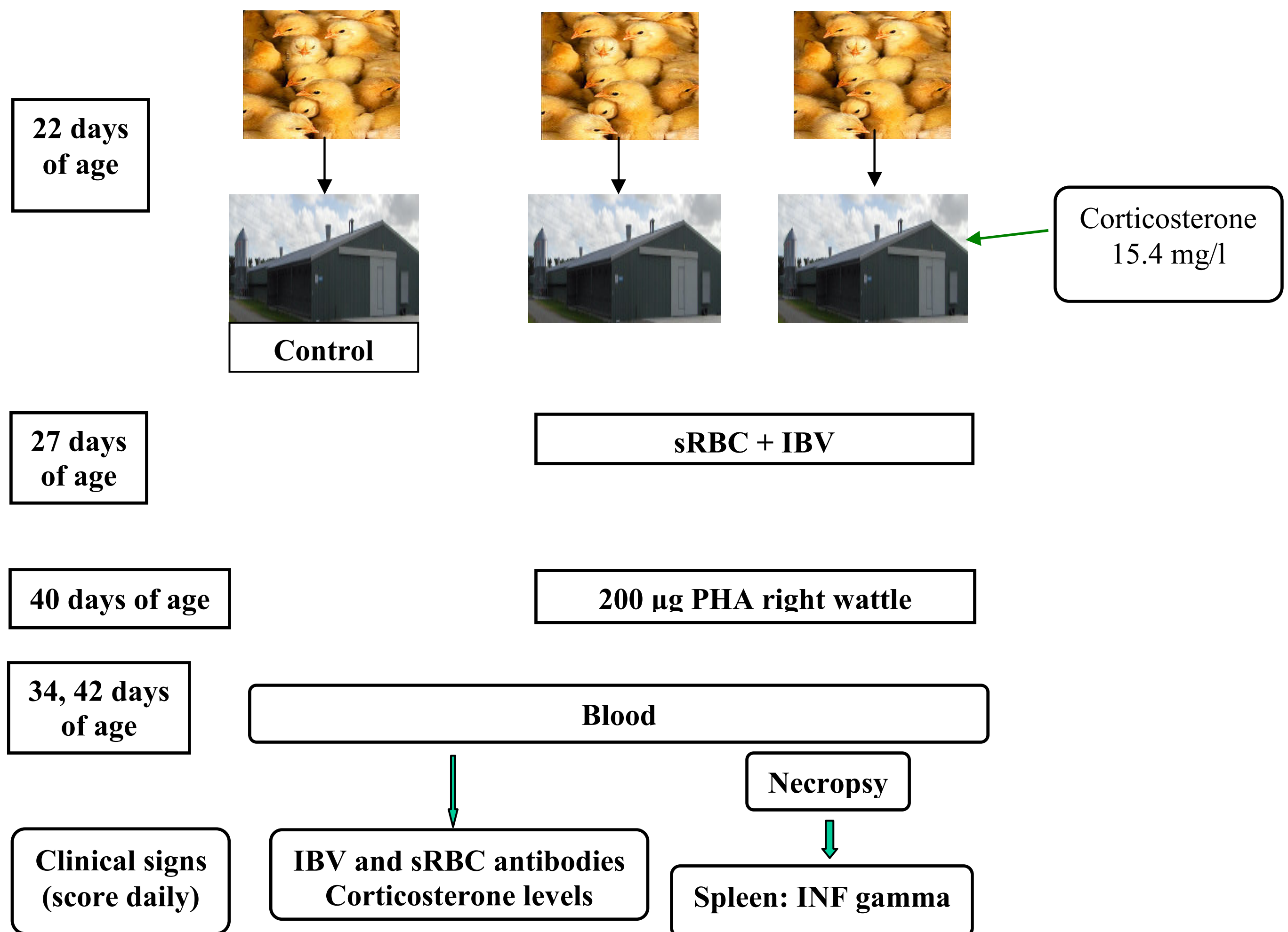


Figure 5.1. Experimental design to test the effect of corticosterone on the immune response in broiler chickens

5.2.3 Infection and immunization

At 27 days of age (5 days after the CORT treatment started) the birds from the second and third group were infected intranasally with IBV (100 µl of 10^6 ELD_{50}/ml, NZ strain C) and were injected in the right breast muscle with SRBC [0.5 ml of packed sheep red blood cells (50% v/v in PBS)]. Groups of birds were euthanased (cervical dislocation), weighed and necropsies were performed at a number of different time points (22, 27, 34 and 42 days of age).

All experimental procedures were conducted in accordance with the requirements of the Lincoln University Animal Ethics Committee.

5.2.4 Clinical signs

For each group, respiratory signs were visually scored (number of birds showing that particular sign) three times a day, using a modification of the method described by Avellaneda et al (1994), as described in Chapter 4. Scores were pooled within groups, and the final score was the mean of the scores on that day.

5.2.5 Lesions

The number of dead birds was recorded daily. Gross lesions in both the dead and euthanased birds (on days 22, 27, 34 and 42 of age) were evaluated following necropsy, using a modification of Avellaneda et al (1994) (liver lesions), Kleven et al (1972) (air-sac lesions), Balog et al (2003) (heart lesions), as described in Chapter 4.

5.2.6 Relative spleen weights

At 42 days of age six birds from each group were euthanasied (by cervical dislocation) and weighed. The ratio (%) between spleen and body weight was calculated (organ weight/body weight x 100).

5.2.7 Tonic immobility (TI)

The time lag between the removal of restraint and the righting response was assessed twice at 23 and 41 days of age. Tonic immobility was achieved by placing birds in a supine position in a U-shaped plastic cradle covered with a cloth and restraining them for 15 seconds, as per Jones and Faure (1981). The time until a full righting response was recorded. Failure to obtain a TI reaction lasting at least 10 seconds resulted in exclusion of the bird from the analysis.

5.2.8 Plasma corticosterone concentration

At 22, 27, 34 and 42 days of age, blood samples (1 ml) were taken from the brachial (wing) vein of 6 birds per treated group, into tubes containing heparin, within three minutes of capture. Samples were centrifuged at 2,300 rpm for 10 minutes, the plasma aspirated and stored at -70°C. Plasma CORT concentration was measured by radioimmunoassay (RIA), at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, NZ (Littin and Cockrem 2001).

5.2.9 Determination of antibody titres

Blood samples (1 ml) were collected from the brachial vein, at 1, 14, 22, 27, 34 and 42 days of age to measure serum antibody levels against IBV and/or SRBC. Antibody production against sheep red blood cells SRBC was measured using a haemagglutination test as described by Wegmann and Smithies (1966). Antibody titres to IBV were determined by Tegel Foods Ltd, New Plymouth, using a commercially available ELISA kit (TropBio Pty Ltd, Queensland, Australia) containing the Vic S strain of IBV, as per Chapter 4.

5.2.10 Interferon gamma

Six birds from each of the three groups were slaughtered on days 22, 27, 34, and 42 in order to assess the levels of interferon gamma (IFN γ) produced by splenic lymphocytes cultured *in vitro* (Lambrecht et al 2000), as per Chapter 4.

5.2.11 Intradermal hypersensitivity

At 40 days of age the birds from the second and third groups were injected in the right wattle with 200 µg of phytohaemagglutinin (PHA) in 0.1 ml of sterile pyrogen-free physiologic saline solution as described by El-Lethey et al (2003). The left wattle was injected with saline

solution as a control. The wattle thickness of each bird was measured with a dial micrometer (Combiice, Mitutoyo, Japan) 24 h later, as described by Tella et al (2002). The response was recorded in millimeters as the difference between the PHA response (right wattle) and the saline response (left wattle), 24 h after injection.

5.2.12 Statistical analysis

For relative spleen weights, antibody levels, IFN γ production, PHA skin reaction, TI and CORT concentrations, treatments were compared by one way ANOVA using MINITAB Statistical Software (Minitab Inc, USA). Where the ANOVA F test was significant across all treatments, differences between the means were determined using the value of the Least Significant Difference (LSD), where LSD equals smallest difference between two means that could be deemed as significant. Assessment of independence between discrete variables (number of birds showing clinical signs and lesions) was carried out using Fisher's exact test.

5.3 Results

5.3.1 Dose effect of corticosterone (pilot trial)

Plasma CORT concentration peaked after 2 days of ingestion and then decreased (Figure 5.2).

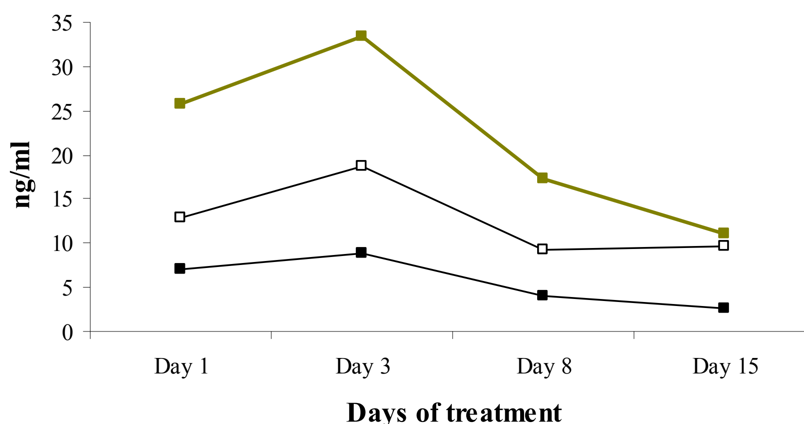


Figure 5.2. Plasma CORT concentrations in chickens untreated (■) or treated with CORT via the drinking water at 15.4mg/l (■) or 7.7mg/l (□)

Both CORT treatments (15.4mg/l and 7.7mg/l) produced significant immunodepression ($p<0.001$) in comparison with the untreated birds, as reflected in the lower production of haemagglutinating antibodies (Figure 5.3).

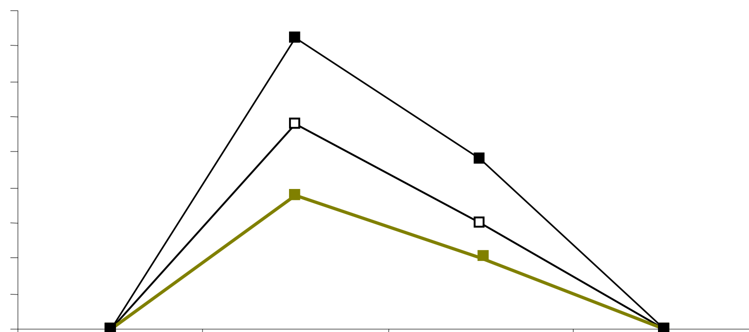


Figure 5.3. Haemagglutinin titre to SRBC measured in birds treated with 15.4 mg/l of CORT (■), 7.7 mg/l of CORT (□) or untreated (■)

5.3.2 Clinical signs

In the main trial there was no effect of corticosterone treatment on the proportion of IBV-challenged birds showing clinical signs (Figure 5.4), although there was a tendency for the clinical signs to persist with CORT treatment.

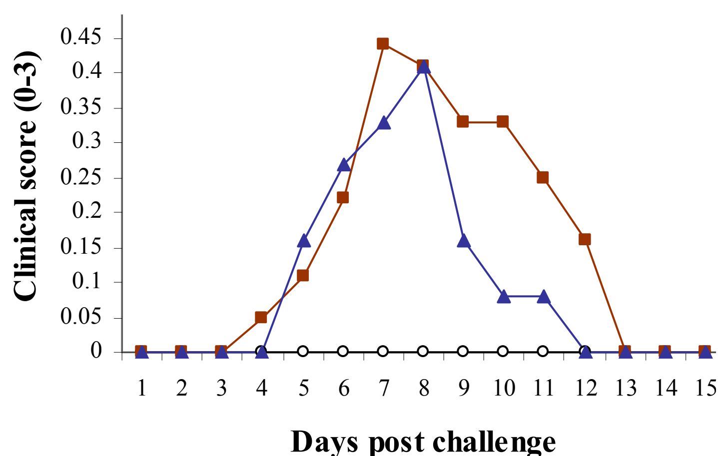


Figure 5.4. Mean clinical score (0-3) of birds under CORT treatment and challenged with IBV (■), untreated with CORT and challenged with IBV (▲), and control (untreated with CORT and no challenge) (○)

5.3.3 Lesions

After 5 and 13 days the CORT treated birds had more hepatic alterations (e.g., enlarged with rounded borders,, fatty infiltration) than untreated birds (p = 0.061 and 0.002, respectively) (Figure 5.5). The CORT treated birds had an increase of intra-abdominal fat (Figure 5.6).

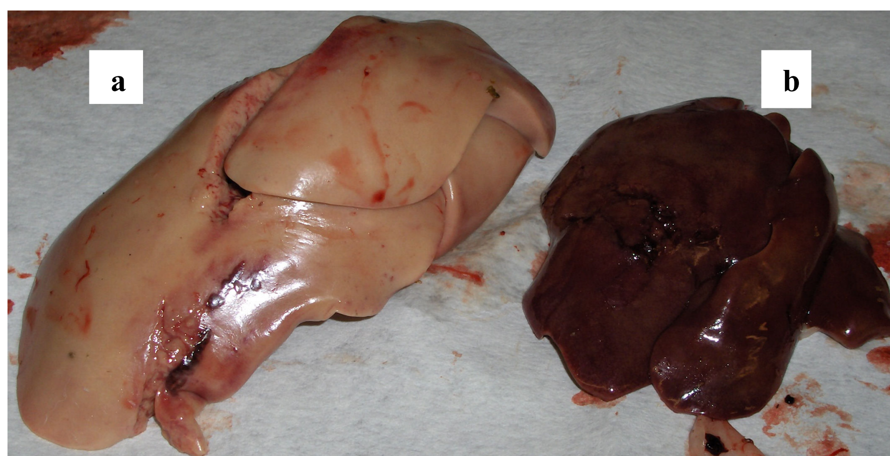


Figure 5.5. (a) Fatty and enlarged liver from a bird under CORT treatment, (b) liver from an untreated bird

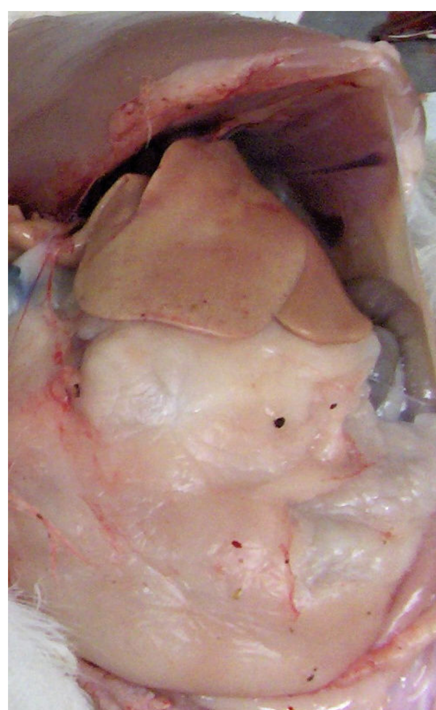


Figure 5.6. Large accumulation of fat in the abdominal cavity of a CORT- treated bird

5.3.4 Body weight and relative spleen weights

At 42 days of age the CORT treated birds had significantly lower body and relative spleen weights ($p<0.01$) than untreated birds (Figure 5.7) (Appendix V)

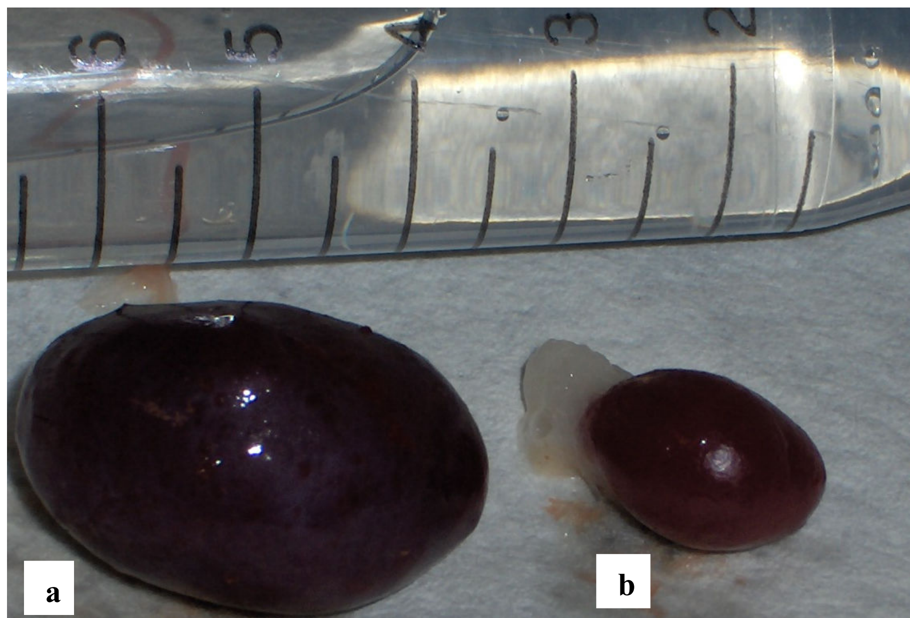


Figure 5.7. (a) Spleen from an untreated bird, (b) spleen from a bird treated with CORT for 22 days. Graduation from 2-3 mls equates to 7 mm)

Table 5.1. Statistical differences between relative spleen weights (grams) (at 42 days of age), determined by LSD. Different letters indicate $p<0.05$

	Mean	
Control group	0.09	b
Group of birds challenged but not treated with CORT	0.08	b
Group of birds challenged and treated with CORT	0.05	a
LSD 5%	0.02	
F test	$p<0.01$	

5.3.5 Tonic immobility (TI) and plasma CORT concentration

At 23 days of age (first day of CORT treatment) there were no differences in TI indices between the three groups, but at 41 days of age the birds under CORT treatment (for 19 days) had higher TI indices ($p<0.05$) than the untreated birds (Table 5.2).

Table 5.2. Statistical differences between the means of TI (41 days of age), as determined by LSD. Different letters indicate $p<0.05$

	Mean	
Control group	273	a
Group of birds challenged but not treated with CORT	353	a
Group of birds challenged and treated with CORT	673	b
LSD 5%	146	
F test	**P<0.001**	

At 34 and 42 days of age, the group of birds treated with CORT had a significantly higher mean of plasma corticosterone concentration ($p<0.05$) than birds not treated with CORT (Figure 5.8).

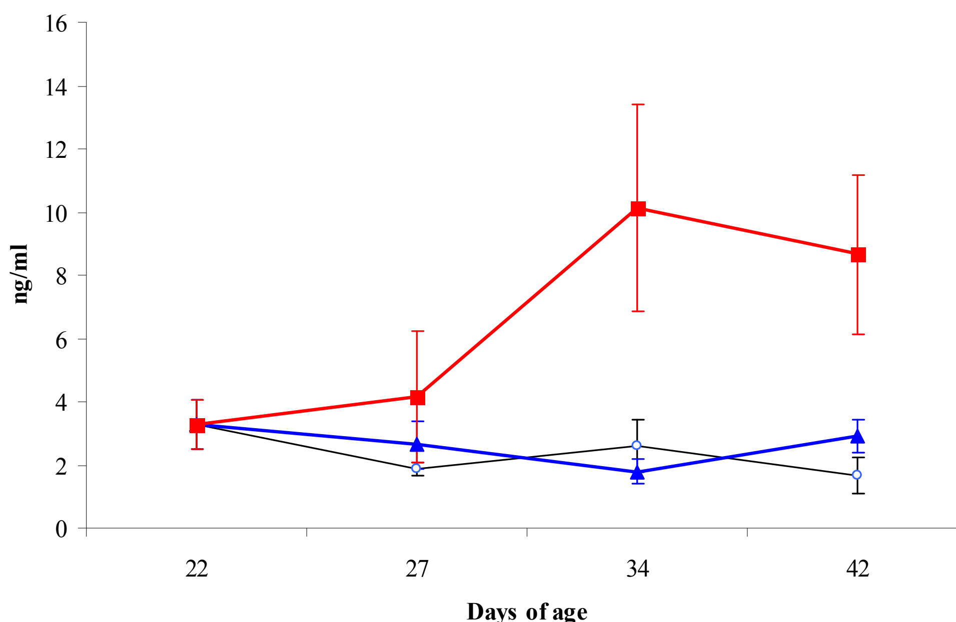


Figure 5.8. Mean plasma levels of corticosterone concentrations of chickens treated with CORT and challenged (■), untreated with CORT but challenged with IBV (▲), and controls (○), untreated with CORT and not challenged with IBV.

5.3.6 Humoral immune response

At day 1 of age the birds had a high titre of antibodies against IBV (data not shown), which progressively declined to zero at 27 days when IBV was administered intranasally and SRBC were injected. Following IBV challenge (from 27 to 42 days of age) the birds under CORT treatment showed a significant ($p<0.001$) increase in antibody titre against IBV in comparison to the birds not treated with CORT (Figure 5.9).

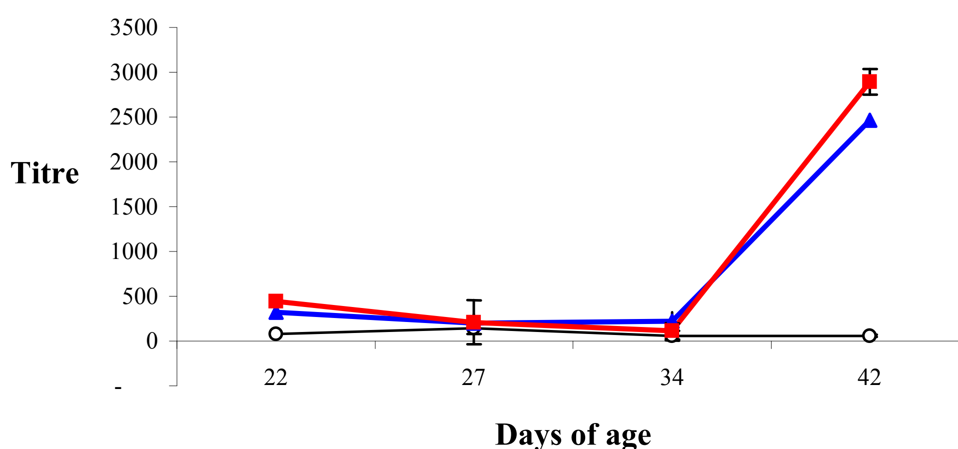


Figure 5.9. Antibody titre to IBV measured by ELISA from chickens challenged with IBV and either treated with CORT (■), or not treated with CORT (▲), or were controls - neither challenged with IBV nor treated with CORT (○)

Table 5.3. Statistical differences between the increase of antibody titre to IBV (day 27 to 42 of age), determined by LSD. Different letters indicate $p<0.05$

	Mean	
Control group	-87	**a**
IBV challenged but not treated with CORT	2261	**b**
IBV challenged and treated with CORT	2671	**c**
LSD 5%	257	
P value	**<0.001**	

At day 7 after challenge (34 days of age) the chickens under CORT treatment had a significantly higher antibody titre against SRBC than the birds not treated with CORT (Figure 5.10) (Table 5.4).

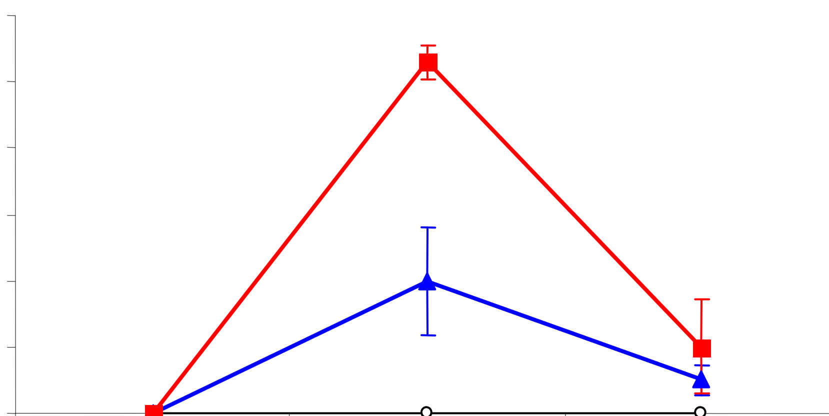


Figure 5.10. Haemagglutinin titres to SRBC measured in birds under CORT treatment and injected with SRBC (■), not treated with CORT and injected with SRBC (▲), and controls (○) neither treated with CORT nor SRBC

Table 5.4. Statistical differences between the means of haemagglutinin titres to SRBC (eight days post challenge), as determined by LSD. Different letters indicate $p<0.05$

	Mean	
SRBC injected but not treated with CORT	2	**a**
SRBC injected and treated with CORT	5.3	**b**
LSD 5%	1.88	
P value	**p=0.003**	

5.3.7 Cell mediated immune response

The levels of IFNγ released by splenic lymphocytes after *in vitro* stimulation with IBV antigen were higher in the CORT treated group seven days after challenge ($p<0.001$) in comparison with birds not treated with CORT but infected with IBV. Fifteen days after IBV

challenge the levels of IFNγ were significantly greater ($p<0.05$) in the birds not treated with CORT compared with those treated with CORT (Figure 5.11) (Table 5.5).

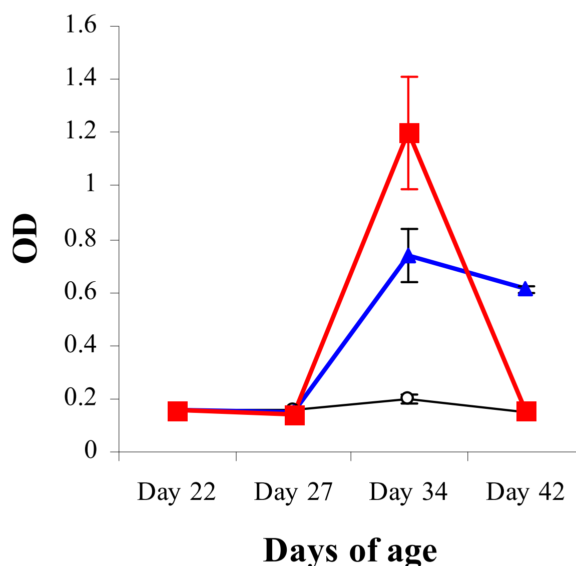


Figure 5.11. Levels of IFNγ (as optical density units) released from spleen cells stimulated with IBV antigen and measured by ELISA, from chickens treated with CORT (■), untreated with CORT (▲), and control (○) – neither treated with IBV nor CORT

Table 5.5. Statistical differences between the mean levels of IFNγ released from spleen cells stimulated with IBV antigen and measured by ELISA (OD) (7 and 15 days post challenge), as determined by LSD. Different letters indicate $p<0.05$

	Mean at 34 days of age (7 days post challenge)	
Control group	0.20	**a**
IBV challenged but not treated with CORT	0.76	**b**
IBV challenged and treated with CORT	1.22	**c**
LSD 5%	0.41	
F test	**p<0.001**	
	Mean at 42 days of age (15 days post challenge)	
Control group	0.16	**a**
IBV challenged but not treated with CORT	0.61	**b**
IBV challenged and treated with CORT	0.17	**a**
LSD 5%	0.03	
F test	**p<0.001**	

The elicited skin reaction to PHA showed that the birds treated with CORT had a significantly smaller ($p<0.001$) reaction (right wattle) (Figure 5.12) (Table 5.6), compared with the control (PBS), irrespective of IBV challenge.

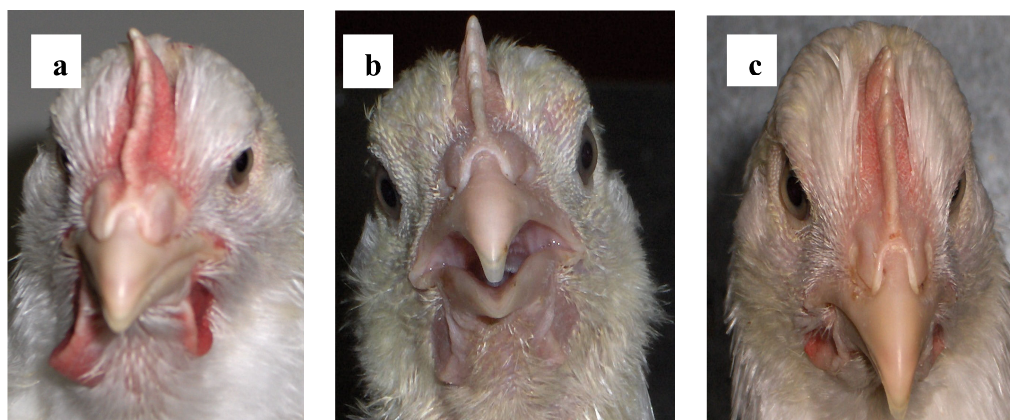


Figure 5.12. Reaction to PHA injection (right wattle) of birds not treated with CORT (a, b), as compared to a bird treated with CORT (c)

Table 5.6. Statistical differences between the means of the PHA reaction (mm), as determined by LSD. Different letters indicate $p<0.05$

	Mean	LSD 5%	P value	
Control group	3.47			**b**
IBV challenged but not treated with CORT	3.39			**b**
IBV challenged and treated with CORT	1.94			**a**
		0.72	**p<0.001**	

5.4 Discussion

It has been reported that certain stressors can enhance or suppress the immune response in birds, for example, feed restriction (Hangalapura et al 2005), high temperatures (Heller et al 1979; Regnier et al 1981), low temperatures (Subba Rao and Glick 1977; Hangalapura et al 2003) and it is assumed that these changes are attributable to CORT released by the adrenal gland in response to the stress. Adrenalectomy eliminates such stress-induced changes (Dhabhar 2003). In this trial, the oral administration of low physiological doses of oral

corticosterone, as compared to higher parenteral pharmacological doses administered in most research trials [(680 micrograms/kg body weight per day, Bartov (1982)], was an attempt to mimic a period of protracted stress. It was important to produce a level of CORT in the serum (>8ng/ml),similar to that found during hypothermic conditions in a previous experiment (Chapter 4).

To our knowledge, this is the first study showing that low levels of exogenous CORT can produce an enhancement in the immune response of chickens. The humoral response to IBV (2 weeks post-challenge) and SRBC *in vivo* and the production of INFγ *in vitro* (1 week post-challenge) was enhanced in birds treated with CORT, in comparison to the control group. Previous studies have highlighted either the general immunosuppressive effects of CORT in birds (El-Lethey et al 2003; Post et al 2003) or lack of effect (Donker and Beuving 1989). However, in this trial the birds treated with CORT showed a decreased skin reaction to PHA in agreement with other studies (El-Lethey et al 2003; Corrier et al 1990). This contradictory response: enhancement (IFN γ and IBV antibodies) and depression (PHA) of immunity could be attributable to the different responses generated. That is, a highly specific recall response (acquired) to an endemic pathogen such as IBV *versus* a non-specific, primary, response to injected PHA.

It is likely that the effects of CORT on the immune response depend on how it was administered, the amount administered, the temporal association between administration of the glucocorticoid and exposure to other environmental insults and the duration of the CORT treatment (Pruett et al 1993; Hangalapura et al 2004b). For example, birds injected with SRBC three days after the start of treatment with corticosterone showed immunodepression (Post et al 2003), but a stress situation (high temperature) occuring later after challenge, enhanced the immune response (Heller et al 1979; Regnier et al 1981).

In the present trials, utilizing the same method of CORT administration and antigen (SRBC) challenge there was an obvious immunological difference that depended on timing or dose. In the pilot trial, the humoral response to injected SRBC was depressed when the birds had two days prior exposure to exogenous corticosterone. However, when the birds had five days prior exposure there was immune enhancement to both test antigens (SRBC and IBV) given by different routes (intramuscular injection and intranasal aerosol, respectively). It should be noted that in the pilot trial plasma CORT concentration was shown to be very high at the moment of challenge (Figure 5.2) [26.7ng/ml (birds drinking 15.4 mg/l) and 18.7 ng/ml (birds drinking 7.7 mg/l)] then it decreased to 5.6 ng/ml (birds drinking 15.4 mg/l) and 4 ng/ml (birds drinking 7.7 mg/l), after fifteen days of oral administration. In the study where Post et al (2003) described immunodepression the level of CORT in plasma was also very high at the moment of challenge [35 ng/ml (birds drinking 20 mg CORT/l)]. The lower levels of plasma CORT concentration at the moment of the challenge in the main experiment (4.14 ng/ml) could have acted as booster to the immune response as regular mild stress such as moderate intensity exercise has been associated with reduced severity of infections and improved antibody responses in mice (Cannon and Kluger 1984). In contrast, a single and strenuous exercise event has been linked to increased susceptibility to infection and reduced immune functions (Davis et al 1997).

Alternatively, the interval between administration of CORT and challenge with antigen (2 days in pilot trial versus 5 days in the main trial) may have been critical to directing immunocompetency. It has been reported that exposure of humans to cortisol for up to a week before challenge with endotoxin enhances TNF-α and IL-6 levels, whereas cortisol at the time of or after endotoxin suppresses the cytokine response (Barber et al 1993). The resultant levels of plasma cortisol achieved in that study at the time of endotoxin challenge, were unknown. The reason why the body responds to a mild stress or low levels of corticosterone

producing enhancement of the immune function may have some 'survival logic' given that one of the primary functions of the brain is to perceive stressors, warn of danger and enable an organism to deal with the consequences (Dhabhar 2002). If a bird sees a predator it is expected that in tandem with the preparation to fight or flight, a response of immunoenhancement rather than immunosuppression would be adaptive since wounding and infection are often the results of aggressive encounters in nature (Dhabhar 2002).

The initial place for replication of IBV in the respiratory tract occurs in the epithelial cells of the trachea (Dhinakar Raj and Jones 1997), subsequently a viremia occurs and the virus gets widely circulated to other tissues (McMartin 1993). In this experiment CORT treatment did not affect clinical outcome as measured by respiratory signs. This, despite the enhanced IBV titres and IFN-γ (*in vitro*) production in the CORT-treated birds at 35 and 42 days of age, respectively.

In the current trial, the birds that drank CORT for 20 days had lower body weights and lower relative spleen weights than the birds not treated with CORT. This is in accordance with previous reports in which significantly lower body weights were observed in CORT-treated birds (Hayashi et al 1994; El-Lethey et al 2001; Post et al 2003). The decrease in body weight was associated with reduced muscle mass and was likely to be due to an increase in net muscle protein catabolism (Hayashi et al 1994) or CORT-impaired insulin signalling in the liver and muscle (Dupont et al 1999). Dohms and Metz (1991) and Post et al (2003) reported that CORT treatment of chickens caused splenic atrophy due to lymphoid depletion of the white pulp.

In the present trial the birds under CORT treatment had enlarged fatty livers, attributable to disturbances in lipid metabolism (lipogenesis) in the liver (Puvadolpirod and Thaxton 2000; Post et al 2003; Saadoun et al 1987). In addition, the CORT-treated birds had larger fat

deposits in the abdomen which has also been reported by other authors (Saadoun et al 1987; Hayashi et al 1994), and is due to the hyperlipaemic effect of the hormone.

Infection with IBV generated an enhanced fear response as measured by TI highlighting the affect of this disease on a chicken's 'wellbeing'. This was significantly increased when IBV-infected birds were treated with CORT indicating the role of the latter, as has been reported by Jones et al (1988) and El-Lethey et al (2003).

In conclusion, the ingestion of low physiological amounts of CORT alters immunity to, and disease outcome from, infections with IBV. While the hormone appeared to exaggerate the effects of IBV (righting reflex and a tendency for increased clinical signs), certain immune functions were enhanced. The response to particulate antigen (SRBC) varied between the 2 different trials which were carried out at different times and with minor differences in management. Thus, a further experiment to test the affect of the duration of administration of exogenous CORT on the immune system was undertaken to clarify this point.

Chapter 6

EFFECTS OF CORTICOSTERONE ON THE IMMUNE RESPONSE IN CHICKENS DEPEND ON DURATION OF ACTION

6.1 Introduction

Current concepts on the role of glucocorticoids such as corticosterone (CORT) in the regulation of inflammatory and immune responses indicate that their role is inhibitory (Wiegers and Reul 1998). However, a large number of studies (e.g., rats, human) have also shown that glucocorticoids exert stimulatory effects on immune function, suggesting that the present concept of the role of glucocorticoids in the immune system is incomplete and needs to be extended (Wiegers and Reul 1998, Sapolsky et al 2000). It is believed that one factor influencing the balance between inhibition or enhancement is the amount of corticosterone produced by a stressor and/or its duration (Davis et al 1997). It has also been proposed that the time between the release or exposure to CORT and the timing of the challenge can change the immune response (towards inhibition or enhancement) (Dhabhar 2002). For example, exposure of humans to cortisol for up to a week before challenge with endotoxin enhances TNF-α and IL-6 levels, whereas cortisol given at the time of, or after, endotoxin suppresses the cytokine response (Barber et al 1993). In rats, pre-exposure to CORT *in vivo* or *in vitro* enhanced mitogenesis of rat splenic lymphocyte mitogenesis after short term pre-exposure to corticosteroid *in vitro* (Wiegers et al 1993; Wiegers et al 1994). In chickens it has been reported that CORT added to feed or water 1 or 2 days before immunization decreases humoral immunity (Gross et al 1980; Post et al 2003). In a previous experiment (Chapter 4), we have shown that a combination of low temperature and IBV infection caused a significant increase of serum CORT. In addition, we have found that birds exposed for 5 days to

physiological levels of CORT before challenge had an enhanced immune response (Chapter 5).

It was therefore hypothesized that the effect of CORT on immune response may depend on the duration of the stress event. In the present experiment, the duration of the stress was to be mimicked by the oral administration of CORT given at different time points and relevant immune responses to antigens assessed.

6.2 Materials and Methods

6.2.1 Animals and housing conditions

Two hundred day-old broiler chickens (Cobb breed) were obtained from a commercial hatchery. The birds were placed in cages and provided with *ad libitum* access to a commercial ration and drinking water until 50 days of age when the trial was terminated. For the first 3 weeks of age the birds had the same light and temperature programme as in Chapter 4; a decreasing light schedule of (23h → 16h per day), and temperature (30°C → 20°C). At 23 days of age the birds were randomly assigned to one of four treatment groups (n=50): group 1 [not challenged with IBV but treated with CORT (14.7 mg/l) in the drinking water from day 23], group 2 [challenged with IBV, injected with SRBC and PHA but not treated with CORT], group 3 [challenged with IBV, injected with SRBC and PHA and exposed to CORT (14.7 mg/l) from the day 23 of age (5 days before challenge)], and group 4 [challenged with IBV, injected with SRBC and PHA and exposed to CORT (14.7 mg/l) from day 26 of age (2 days before challenge)], (Figure 6.1). Each treatment group was housed in isolated rooms with separate air, food and water sources.

6.2.2 Experimental infection, immunization and blood sampling

At 28 days of age all birds in groups 2, 3 and 4 were infected (challenged) intranasally with 100 µl of 10^6 EID_{50}/ml of IBV (NZ strain C). At the same time, 0.5 ml of packed sheep red blood cells (50% v/v in PBS) was injected into the right breast muscle of all birds in groups 2, 3 and 4. Additionally, at 45 days of age, 8 birds from groups 2, 3 and 4 were injected in the right wattle with 200 µg of PHA in 0.1 ml of sterile pyrogen-free physiological saline solution, while the left wattle was injected with saline as a control. The wattle thickness of each bird was measured with a dial micrometer (Combiice, Mitutoyo, Japan) 24 h later, as described by Tella et al (2002). The response was recorded (mm) as the difference between PHA response (right wattle) and the saline response (left wattle) as described in Chapter 5. Blood samples (1 ml) were collected from the brachial vein within 3 minutes of capture at 14, 23, 28, 36 and 43 days of age. Samples were centrifuged at 2,300 rpm for 10 minutes and the plasma and serum aspirated and stored at -70°C (Figure 6.1).

All experimental procedures were conducted in accordance with the requirements of Lincoln University Animal Ethics Committee.

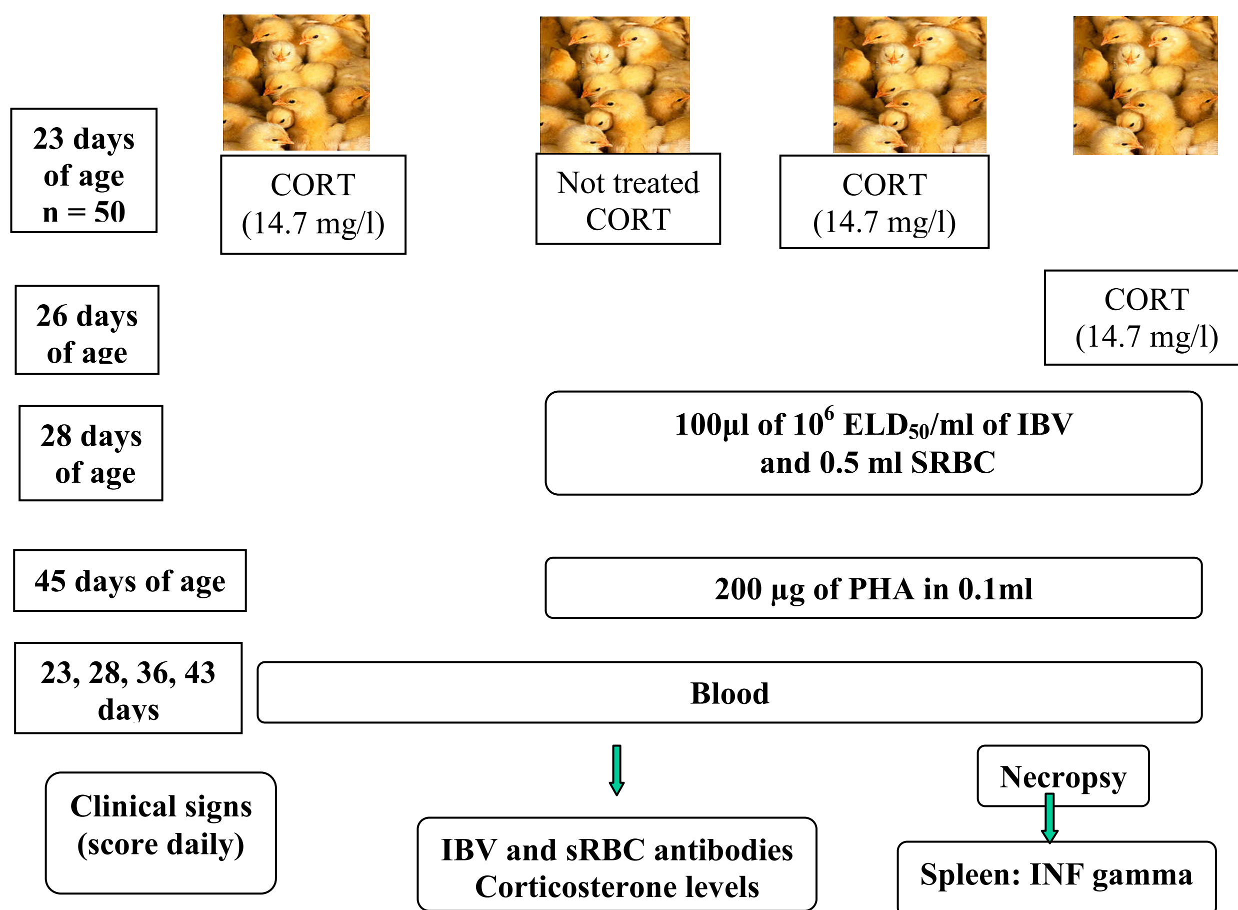


Figure 6.1. Experimental design to test the effects of corticosterone on the immune response in chickens

6.2.3 Clinical signs and lesions

The methodology used for the evaluation of the clinical signs and lesions was the same as that described in the Chapter 4 and 5 and as described by Avellaneda et al (1994), with some modifications (scores 0-3 instead of 1-4).

The air-sacs lesions were scored as described by Kleven et al (1972), liver lesions as described by Avellaneda et al (1994), and heart lesions as described by Balog et al (2003), with modifications as outlined in Chapter 5.

At slaughter (50 days of age) body and spleen weights were measured and relative spleen weights calculated (spleen/body weight x 100).

6.2.4 Tonic immobility (TI)

The righting response was assessed twice, at 22 and 49 days of age. Tonic immobility was achieved by placing birds in a supine position, in a U-shaped plastic cradle, covered with a towel, as described in the Chapter 5.

6.2.5 Corticosterone levels in drinking water and plasma

The CORT levels were measured by radioimmunoassay (RIA), at the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, NZ (Littin and Cockrem 2001).

6.2.6 Determination of antibody titres

Antibody (IgG) titres to IBV were measured from blood serum were collected on days 1, 14, 23, 28, 36, 43, and 50 of age by Tegel Foods Ltd, New Plymouth, using a commercially available ELISA kit (TropBio Pty Ltd, Queensland, Australia), containing the IBV Vic S strain as capture antigen. Antibody production against SRBC was measured using a micro-haemagglutination test (1:2 dilution), as described by Wegmann and Smithies (1966) and results were expressed as the reciprocal of the highest dilution of serum showing specific agglutination with antigen.

6.2.7 Interferon gamma (INFγ)

Twelve birds from each of the four groups were slaughtered on days 23, 28, 36, and 43 in order to assess the levels of INFγ produced by splenic lymphocytes cultured *in vitro* (Lambrecht et al 2000), as per Chapter 4.

6.2.8 Statistical analysis

Assessment of independence between discrete variables (number of birds showing clinical signs and lesions) was carried out using Fisher’s exact test (2 x 2 table).

Relative spleen weights, antibody levels, INFγ production, PHA skin reaction, and TI and CORT levels among treatment groups, were compared by one way ANOVA (MINITAB Statistical Software, Minitab Inc, USA). Where the ANOVA F test was significant, differences between the means were determined using the value of the Least Significant Difference (LSD).

6.3 Results

6.3.1 Clinical signs and lesions

At no time after the challenge with IBV was there any statistically significant evidence that the proportion of CORT treated birds showing clinical signs differed from the proportion of non CORT-treated chickens (Figure 6.2).

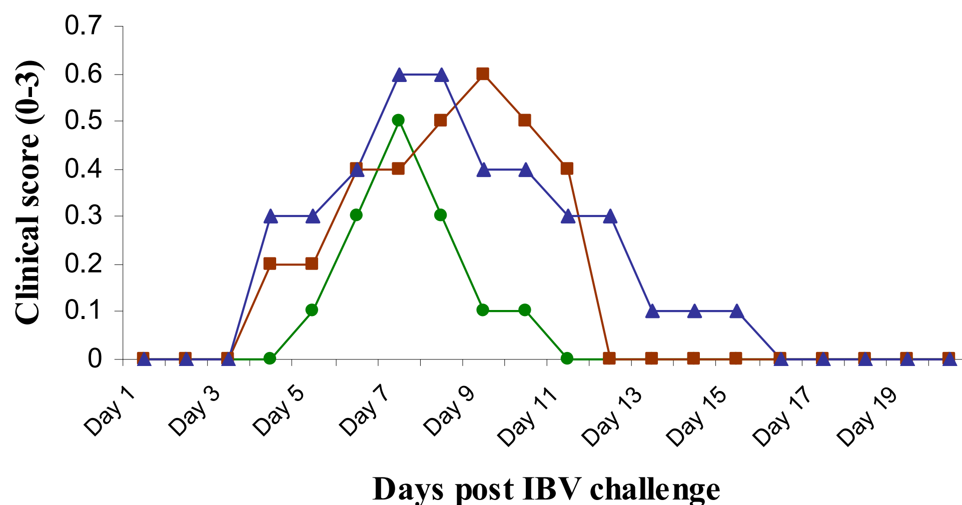


Figure 6.2. Mean clinical score (0-3) of birds (n =10) under CORT treatment for two days before challenge with IBV (▲), treated with CORT for 5 days before challenge with IBV (■), and not treated with CORT but challenged with IBV (●)

Birds in the three CORT treatment groups, developed a significantly higher proportion of hepatic lesions ($p<0.05$) compared with the birds not treated with CORT but challenged with IBV, however there were no differences among the different CORT treatment groups (Figure 6.3).

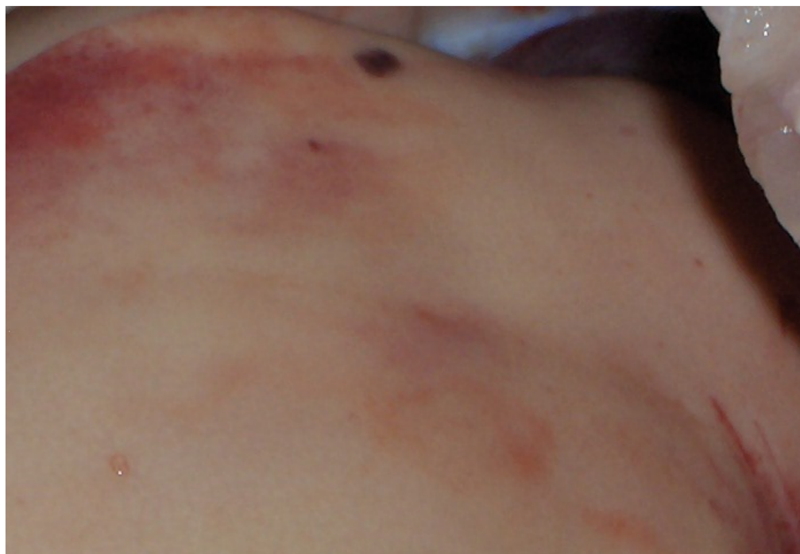


Figure 6.3. Liver with haemorrhages, found in bird under CORT treatment from 23 days of age,

6.3.2 Body and spleen weights

At 50 days of age the birds under CORT treatment (treatments groups 1, 3 and 4) had significantly lower relative spleen weights ($p<0.01$) compared with birds not treated with CORT. The mean relative spleen weights of birds that started the CORT treatment at 23 days of age was lower ($p<0.05$) than that of birds starting the CORT treatment at 26 days of age.

Table 6.1. Body and spleen weights from birds untreated with CORT but challenged with IBV (IBV), treated with CORT from day 23 but not challenged with IBV (CORT 23), treated with CORT from day 26 of age and challenged with IBV (IBV+CORT 26) and birds treated with CORT from day 23 and challenged with IBV (IBV+CORT 23)

Treatment	Body Weight	Spleen Weight	%
CORT 23	1380	0.77	0.055
CORT 23	1370	0.74	0.054
CORT 23	1340	0.8	0.059
CORT 23	1530	1.2	0.078
CORT 23	993	0.42	0.042
CORT 23	916	0.45	0.049
CORT 23	1452	0.98	0.067
CORT 23	1560	1.13	0.072
			0.059
IBV+ CORT 26	1820	0.99	0.054
IBV+ CORT 26	1570	0.87	0.055
IBV+ CORT 26	1352	0.86	0.063
IBV+ CORT 26	1034	0.54	0.052
IBV+ CORT 26	1156	0.65	0.056
IBV+ CORT 26	1910	1.2	0.058
IBV+ CORT 26	970	0.98	0.1
IBV+ CORT 26	1030	0.97	0.094
			0.06
IBV+ CORT 23	2080	1.01	0.048
IBV+ CORT 23	1988	0.89	0.044
IBV+ CORT 23	1307	0.84	0.06
IBV+ CORT 23	1216	0.69	0.056
IBV+ CORT 23	938	0.45	0.047
IBV+ CORT 23	1012	0.65	0.064
IBV+ CORT 23	1345	0.76	0.056
IBV+ CORT 23	969	0.53	0.054
			0.053
IBV	2643	3.57	0.135
IBV	2534	3.2	0.126
IBV	2654	3.48	0.131
IBV	2175	3.17	0.145
IBV	2452	3.26	0.132
IBV	2320	3.05	0.131
IBV	2178	2.97	0.136
IBV	2453	3.03	0.123
			0.132

6.3.3 Tonic immobility and CORT levels

At 49 days of age there were no differences in TI indices between the four treatment groups.

At 28 days of age the birds that started the CORT treatment 5 days before challenge (23 days of age) had a higher ($p<0.01$) mean plasma CORT concentration than the birds not treated with CORT (treatment group 2). At 36 days of age birds treated with CORT (groups 1, 3 and 4) had higher ($p<0.01$) mean plasma CORT concentrations than the birds not treated with CORT (group 2), but there were no differences among the CORT treated groups (groups 1, 3 and 4). At 43 days of age the birds that started CORT treatment at 23 days of age (5 days before challenge) had a higher ($p<0.01$) mean plasma CORT concentration than the other groups (groups 1, 2 and 4) (Figure 6.4).

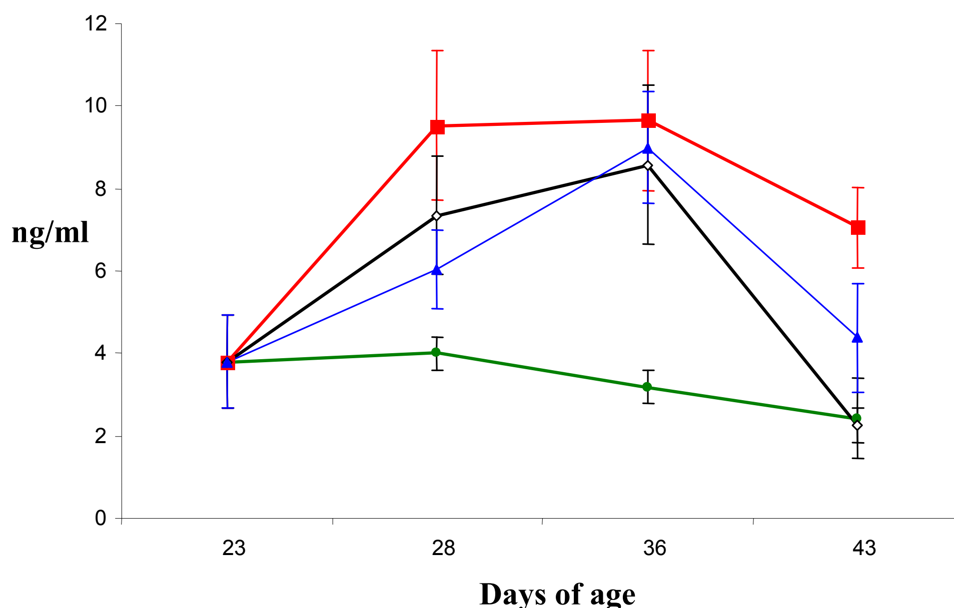


Figure 6.4. Plasma CORT concentration in chickens following CORT treatment from 26 days of age and challenged with IBV (▲), treated with CORT from 23 days of age and challenged with IBV (■), treated with CORT from 23 days of age but not challenged with IBV (○) and birds not treated with CORT but challenged with IBV (●)

6.3.4 Humoral immune response

At 1 day of age the birds had high titres of maternally-derived antibodies against IBV, which had declined to zero at 28 days when IBV was administered intranasally and SRBC was injected. At 43 days of age (15 days post challenge) the birds challenged with IBV had similar IBV titres irrespective of CORT treatment, but at 50 days age all groups had equivalent titres (Figure 6.5). The fact that the control group seroconverted (asymptomatically) indicated a cross contamination event had occurred between isolation rooms.

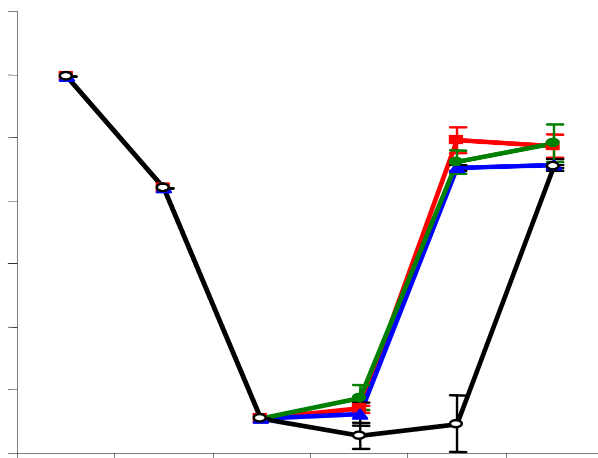


Figure 6.5. Antibody titre to IBV (ELISA) in chickens following CORT treatment from 26 days of age and challenged with IBV (▲), treated with CORT from 23 days of age and challenged with IBV (■), treated with CORT from 23 days of age but not challenged with IBV (○) and birds not treated with CORT but challenged with IBV (●)

By day 8 after injection with SRBC (36 days of age) the chickens treated with CORT from 23 days of age (5 days before challenge) had a higher ($p<0.01$) mean antibody titre than birds not treated with CORT, which in turn developed a higher ($p<0.05$) humoral immune response than the birds treated with CORT from 2 days before challenge (26 days of age). At 15 days post challenge there were no significant differences between treatments (Figure 6.6).

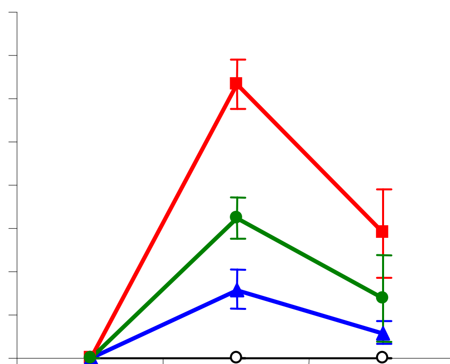


Figure 6.6. Haemagglutinin titre to SRBC measured in chickens treated with CORT from 2 days before injection with SRBC (at 28 days of age) (▲), treated with CORT from 5 days before injection with SRBC (■), treated with CORT at 23 days of age but without injection with SRBC (○) and birds not treated with CORT but with injection with SRBC (●)

Note: the variability of HA titres between experiments (chapters 5 and 6). This was due to a different source of sheep red blood cells which varied with respect to their antigenicity.

6.3.5 Cell mediated immune response

At 36 days of age (8 days post challenge) the levels of INFγ released by splenic lymphocytes after *in vitro* stimulation with IBV antigen were higher ($p<0.05$) in the group of birds that had been exposed to CORT from 5 days before challenge (23 days of age) in comparison with the birds exposed to CORT from two days before challenge (26 days of age). At 15 days post challenge the birds not treated with CORT had higher INFγ levels ($p<0.01$) than the birds treated with CORT irrespective of duration of treatment (Figure 6.7).

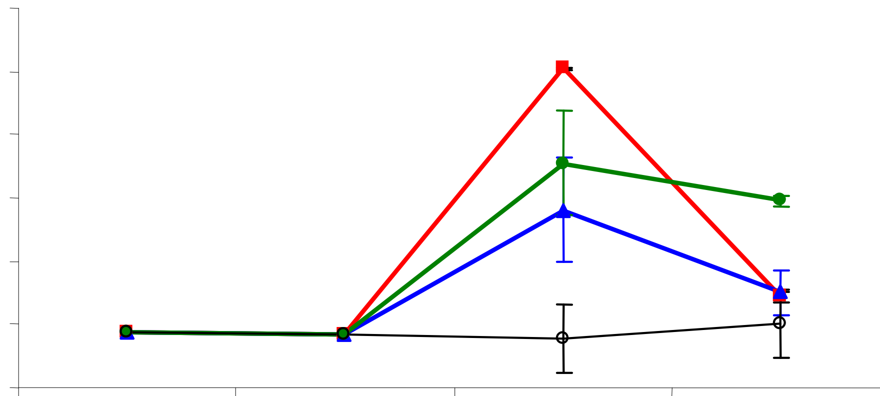


Figure 6.7. Levels of interferon gamma released from spleen cells stimulated with IBV antigen and measured by ELISA in chickens treated with CORT from two days before challenge with IBV (▲), treated with CORT from 5 days before challenge with IBV (■), treated with CORT at 23 days of age but not challenged with IBV (○) and not treated with CORT but challenged with IBV (●)

The skin reaction produced by the intradermal injection of PHA (injected 24 hours previously at 49 days of age) in the birds from the groups treated with CORT, either 2 or 5 days pre-challenge, had smaller ($p<0.001$) reactions (right wattle) than birds infected with IBV but not treated with CORT.

Table 6.2. The skin reaction produced by the intradermal injection of PHA into the wattles of the birds from groups treated with CORT 2 days pre-challenge with IBV (IBV+CORT 26), 5 days pre-challenge (IBV+CORT 23), and not treated with CORT (IBV).

Treatment	Left Wattle	Right Wattle	Difference
IBV+CORT 23	4.69	11.28	2.4
IBV+CORT 23	5.1	10.71	2.1
IBV+CORT 23	2.28	8.59	3.76
IBV+CORT 23	1.92	8.09	4.21
IBV+CORT 23	1.86	8.08	4.34
IBV+CORT 23	2.87	9	3.13
IBV+CORT 23	3.3	9.7	2.93
IBV+CORT 23	3.24	10.08	3.11
			3.24
IBV	1.9	10.98	5.77
IBV	1.92	12.67	6.59
IBV	1.09	5.09	4.66
IBV	1.41	7.6	5.39
IBV	1.27	4.34	3.41
IBV	1.27	5.33	4.19
IBV	2.08	10.2	4.9
IBV	2.1	11.09	5.2
			5.01
IBV+CORT 26	2.05	7.34	3.58
IBV+CORT 26	2	3.2	1.6
IBV+CORT 26	1.69	4.55	2.69
IBV+CORT 26	1.77	5.53	3.12
IBV+CORT 26	3.05	6.22	2.03
IBV+CORT 26	1.69	5.63	3.3
IBV+CORT 26	1.72	4.62	2.68
IBV+CORT 26	1.65	4.44	2.69
			2.71

Table 6.3. Summary of the effects of CORT on the immune response of chickens treated with CORT from 2 days before challenge with IBV and injection with SRBC (CORT 26 IBV), treated with CORT from 5 days before challenge with IBV and injection with SRBC (CORT 23 IBV), treated with CORT at 23 days of age but without challenge with IBV or injection with SRBC (CORT), and birds not treated with CORT but challenged with IBV and injected with SRBC (IBV). Different letters indicate $p<0.05$.

	CORT 23 IBV	**CORT 26 IBV**	**IBV**	**CORT**
Hepatic lesions	+++b	+++b	+a	+++b
Humoral response SRBC 8 days post challenge	+++c	+a	++b	
Humoral response SRBC 15 days post challenge	++a	++a	++a	
Humoral response IBV 15 days post challenge	+++a	+++a	+++a	
Humoral response IBV 22 days post challenge	+++a	+++a	+++a	+++a
Cell mediate response IBV (IFNγ) 8 days post challenge	+++b	++ab	++a	
Cell mediated response IBV (IFNγ) 15 days post challenge	+a	+a	++b	
Skin reaction PHA 24 hours post injection	+a	+a	+++b	

6.4 Discussion

It is clear from these results that the longer duration (5 days *vs* 2 days) of exposure to elevated CORT levels in blood prior to the immune challenge caused an enhancement of the resultant immune response. This is consistent with the view that corticosteroids can exert distinct, seemingly paradoxical, effects on cytokine expression, cytokine receptor expression and cytokine-regulated biological response (Wiegers and Reul 1998). The majority of the studies on corticosteroids have focused on the immunosuppressive actions of these stress hormones. It is paradoxical that organisms should have evolved mechanisms to suppress immune

function at a time when an active immune response may be critical for survival (Dhabhar 2002). While on the one hand, stress is thought to suppress immunity and increase susceptibility to infections and cancer (Baltrusch et al 1991), on the other hand it also exacerbates autoimmune and inflammatory diseases (Mei-Tal et al 1970) although glucocorticoids are administered clinically to treat these diseases (Schleimer et al 1989). Keeping these issues in mind, and based on the earlier studies (Chapter 5), it was hypothesized in this experiment that CORT may either enhance or suppress immune function depending on the timing of the administration.

In birds where CORT has been administered only inhibitory immune effects have been demonstrated (Dohms and Metz 1991; El-Leyhey et al 2003, Post et al 2003). In the previous experiment (Chapter 5) it was found that the immune system of the birds responded in different ways, depending of the duration of the CORT treatment (2 days before challenge *vs* 5 days before challenge). However, the pilot and main trial were carried out at different times. In order to confirm these results CORT was administered over two different time intervals prior to challenge with an infectious agent (IBV) in the current experiment. The tests used to measure the immune response and the antigens were the same as used previously.

For the first time in poultry it has been shown in this trial that the time between the beginning of the CORT treatment and the moment of the challenge is crucial (Barber et al 1993; Wiegers et al 1994). Birds that started to drink CORT 5 days before injection with SRBC had an enhancement of the humoral immune response in comparison with the birds that started the treatment 3 days later (2 days before challenge) or the birds that were injected with SRBC but not treated with CORT. Furthermore, the birds that began to drink CORT 5 days before the challenge developed a higher cell mediated immune response as measured by the level of INF-γ released by the splenic lymphocytes after *in vitro* stimulation with IBV, in comparison

with the birds exposed to CORT for two days before challenge. Both were transient effects, apparent 1 week after challenge. In humans, exposure to cortisol (hydrocortisone sodium succinate, 500 mg/500 ml 5% dextrose, given i.v. by volume control pump at a rate of 3 μg/kg/min) for up to a week before a challenge with endotoxin enhances TNF-ά and IL-6 levels, whereas cortisol at the time of or after endotoxin challenge suppresses the cytokine response (Barber et al 1993). Hangalapura et al (2004a) reported that 7 days of cold stress, which has been reported to increased plasma CORT concentration (Luger et al 2003; and see chapter 4), significantly enhanced cellular immunity, whereas 2 days of cold stress treatment had differential effects on the cellular immunity, depending on the line of birds and the time of immunization. The present work indicates that this effect may be due to the direct effects of CORT. The results of this current experiment do not support the support the hypothesis that the immunological actions of the CORT are vested in the basal levels of CORT in blood at the moment of challenge as the blood levels of CORT in those animals treated did not differ significantly. Our data support the belief that a long pre-exposure to physiological levels of exogenous CORT causes adaptation for example, down-regulation of glucocorticoid receptors or alteration of their affinity (Armario et al 1994; Fleshner et al 1995). In an experiment described by Post et al (2003) the immunopressive effects of exogenous CORT were due to very high plasma CORT concentrations (30-35 ng/ml), in comparison to the levels reached in this experiment (9.5 ng/ml). The alterations in the CORT receptors are suggested because removal of CORT via adrenalectomy increases corticosteroid binding globulin (CBG) levels. CBG is a plasma carrier protein important for transporting CORT in a biologically inactive form. CORT that is bound to CBG can not reach many target tissues and can not bind with target receptors (Pardridge 1981). A reduction in the CBG would be expected to produce a measurable increase in unbound or free biologically active CORT (Mendel 1989). The CORT assay used in this experiment measured total CORT so it was not possible to estimate the

amount of free CORT in the plasma. Enhancement of the immune response may occur via an increase in receptor expression on several cell types (e.g., T cells) for IL-1 (Akahoshi et al 1988), IL-2 (Fernandez-Ruiz et al 1989), IL-4 (Paterson et al 1994), IL-6 (Campos et al 1993) and IFN-γ (Strickland et al 1986). Furthermore, CORT administration induces a reduction of blood leukocyte numbers, which reflects leukocyte redistribution from the blood to organs such as the skin, mucosal lining of gastro-intestinal and urinary-genital tracts, lung, liver, and lymph organs (e.g., spleen) where immunosurveillance occurs due to innate and acquired immune mechanisms (Dhabhar 2002). Mechanisms involving leukocyte activation are also enhanced during some stress situations; macrophage phagocytic activity (Lyte et al 1990) would enhance innate immunity whereas increased macrophage antigen presentation (Sadeghi et al 1992) would enhance adaptive immunity.

The skin reaction against PHA injected 24 hours previously (day 49 of age) showed that the birds from the groups treated with CORT either 2 or 5 days pre-challenge had significantly smaller reactions in agreement with our previous studies. This process (skin reaction against PHA) involves a variety of cells such as macrophages, basophils, heterophils, B lymphocytes and cytokines secreted by the local T lymphocytes (Stadecker et al 1977). It is an *in vivo* test that provides a general index of non-specific cell mediated immunity (Tella et al 2002). The other test used in this experiment was an *in vitro*, antigen-specific (IBV) assay that reflects the production of INFγ from T cells present in the spleen that had been previously sensitized (Lambrecht et al 2000). In order to try and elucidate if the lesions in the different tissues were caused by the use of CORT or by IBV, a group of birds were kept isolated from the pathogen (treatment group 1). However by the end of the experiment a contamination event involving IBV had occurred via the ventilation system and the control birds developed antibodies (Figure 6.3) indicating an inadvertent infection had occurred. The birds under CORT treatment developed a significantly higher proportion of hepatic lesions in comparison with

those not treated with CORT irrespective of IBV infection in accordance with previous reports (Saadoun et al 1987; Puvadolpirod and Thaxon 2000; Post et al 2003). The administration of CORT caused splenic atrophy in agreement with Dohms and Metz (1991) and Post et al (2003) and in parallel with the dramatic decrease in INF γ production from spleen cells, 15 days post challenge.

In this study the duration of the tonic immobility did not show significant difference between groups in contrast to the previous experiment (Chapter 5). This response, triggered by physical restraint and characterised by a catatonic-like state of reduced responsiveness, shows large variability between animals and sometimes the results do not agree with other tests (Campo et al 2005) used for measuring animal welfare.

In summary, duration of CORT treatment affects immune outcome. It is not clear whether this is due to temporal effects *per se* or cumulative dose at the time of antigen challenge. It is unknown whether this transient period of immmunoenhancement gives benefits to birds as they adjust homeostatically to their environmental stressors.

Chapter 7

GENERAL DISCUSSION

The poultry industry in New Zealand holds an enviable sanitary status. Major pathogens such as infectious bursal disease virus, chicken anaemia virus and Newcastle disease virus are absent or if present, show a mild pathogenicity or are controlled by limited breeder vaccination. This status can be attributed to progressive biosecurity legislation and practices and the fact that the majority of the poultry industry is controlled by a small number of companies, who use well-designed, rational, vaccinal and therapeutic programmes. Despite the improvements in health and management, diseases such as infectious bronchitis are still putting the sanitary status of poultry flocks in serious risk. In New Zealand, as in many countries, IB shows distinct winter seasonality, despite the flocks being housed in controlled environment sheds. New Zealand, as well as many other developed countries is experiencing a tendency for consumers to want to return to more ‘natural’ husbandry systems for rearing poultry, such as free range chickens. Under this production system, which has few restricting criteria, the birds may face many stressors such as extreme ambient temperatures that can jeopardize their homeostasis and, so, affect their welfare. In New Zealand the free range concept is typically more limited with options to go outside and usually only after 3 weeks of age.

The first experiment described in this thesis was a case control study (Chapter 3), to determine the prevalence of IBV in broilers within the Canterbury province of New Zealand in late winter and spring and to assess whether a particular management practices or environmental factors (risk factors) were related to the presence of IBV. It was found that there was a high (50%) prevalence of IBV, as detected by RT-PCR, in the farms visited in Canterbury, but under the conditions of management in these large commercial units (e.g.,

temperature, inter-current infections, etc), the virus caused mild disease. Environmental changes are the explanation most often used to explain the seasonality of infectious diseases. In this experiment, it was found that relative humidity within the sheds had an inverse relationship (p=0.05; Odds Ratio=0.92) to the presence of the virus, which means that high humidity is unfavourable for the presence or establishment of the virus in the cloaca and trachea. It has been proposed that the drying of mucosal surfaces increases the probability of colonization by microorganisms (Dowell 2001). With airborne human coronaviruses it has been reported that high relative humidity is less favourable to its survival than low humidity (Ijaz et al 1985).

With a possible change to 'free range' management systems one question that arises is, what changes in IB status could occur in New Zealand? It was assumed that extremes (high or low) of ambient temperature would be one of the principal stress situations that the fast growing broilers will face in a "free range" situation and for that reason this stress factor was initially studied.

Previous studies on the effect of temperature on the immune system in birds had showed inconsistent results. Some studies reported that an extreme temperature (high or low) can produce immunosupression but other studies have shown an enhancement of the immune response. The variability between results can be attributed, in part, to the specific immunologic parameters measured, assay techniques and the different breeds of chickens used in the different experiments. In the first experiment to be conducted (Chapter 4), 3 different temperature regimes were chosen bracketing the temperature that the birds could face under field conditions: either high (30 +/- 2°C), or moderate (20 +/- 2°C) or low (10 +/- 2°C), with relative humidity levels of 45-55%. Following infection with IBV, it was found that low temperatures caused an exacerbation of the IBV-like respiratory signs and that high

temperatures caused hepatic lesions. Given the ubiquitous presence of IBV in many poultry sheds the implications for such temperature changes are clear. It is not known what effect short – term temperature fluctuations of the same magnitude (+/- 10 ^{o}C) would have.

By thirteen days after IBV challenge (35 days of age), the mean antibody titre for IBV was lower in birds housed under high ($p<0.05$) temperature compared to the moderate temperature and there was a tendency to have lower antibody levels to IBV in birds housed under the low temperature. The immunosuppressive effects of stress have been hypothesized to be via the production of glucocorticoids, and in this experiment a link between low (but not high) temperature and CORT levels in plasma (8, 15, and 22 days post challenge) was found. Several researchers have demonstrated that stress-induced suppression of immunity can be produced in an adrenal-independent manner, suggesting that a variety of other hormones, neurosecretory products and cytokines may also be involved (Bonneau et al 1993; Keller et al 1983). It has been reported that heat stress decreases the release of T-helper 2 cytokines, that are important for antibody production (Lebman and Coffman 1998), and that heat stress in chickens also stimulates free oxygen radical production, which is known to have several non-specific damaging effects on the immune system (Mujahid et al 2005). Possibly, the requirement of nutrients required to maintain an efficient immune response were not met in the current experiment due to a reduction in feed intake plus low feed digestibility (proteins, fat, starch), and this became evident when the birds are under heat stress (Bonnet et al 1997). Interestingly, the humoral response to the other antigen used (SRBC) was reversed; that is enhancement in the birds housed at high temperature. This enhanced response was similar to observations made by Heller et al (1979) and Regnier et al (1980) following short periods of elevated temperatures and using the same antigen (SRBC). The difference in the humoral immune response to IBV and to SRBC may simply be a reflection of the antibody isotype (IgG vs IgM) detected by the different assays used or it could indicate fundamental

differences in immunity to these antigens. These differences could relate to type of preparation (particulate; small versus large RBC), route of administration (injection vs intranasal) and the presence of inflammation with the IBV challenge. Either way the findings should hint at being cautious when using the terms immunosuppression or enhancement. The other physiological parameters studied such as body weight, haematocrit, and tonic immobility showed that the birds under extreme temperatures (high or low) had to make substantial physiological adjustments in order to return to homeostasis. These adjustments can jeopardize their welfare.

From the time chicks are hatched, throughout their lives, different stressors confront them and threaten their welfare. The physiological regulation of homeostasis is attained by complex endocrine interactions, principally by the hormones secreted from the adrenal gland (Harvey et al 1984). Corticosterone (CORT) is the principal glucocorticoid released by adrenal glands in birds in response to hypothalamic-pituitary activity (Harvey et al 1984). Current concepts on the role of CORT in the regulation of inflammatory and immune response depict its role as being inhibitory. Surprisingly, CORT has been shown to up regulate the expression of various cytokine receptors and express synergism with several cytokines, which is in apparent contrast to the other ‘restraining’ effects of this hormone (Wiegers and Reul 1998). The trial described in Chapter 5 sought to assess the effects of the stress on the immune system by the use of exogenous CORT in the drinking water in an attempt to produce levels of CORT in the blood that would be similar to those found in stress situations. An initial pilot trial was carried out in order to assess the correct amount of CORT to administer via the drinking water for the principal trial. For logistic reasons the time between when the birds started to drink CORT and the time of the IBV challenge, was not the same for both trials (the pilot trial and the main trial). In the pilot trial, when the birds were challenged 2 days after the start of the CORT

treatment, immunosuppression of humoral immunity occurred, in contrast to enhancement of the immune response in the main trial, where the birds were challenged 5 days after the start of the CORT treatment. Because these contradictory results could have been a reflection of different management factors at the different trial times an additional experiment was carried out (Chapter 6). In this experiment IBV infections occurred at 2 or 5 days after the beginning of the CORT treatment. It was found that the time between the CORT treatment and challenge was crucial for determining the immune response (enhancement or suppression), as it is in humans (Barber et al 1993) and rats (Wiegers et al 1994). The birds that started to drink CORT 5 days before being injected with SRBC and challenged with IBV showed an enhancement in the humoral and cellular immune response in comparison to the birds that started the treatment 3 days latter (2 days before challenge). The reasons for this are unknown, but it is understood that the immune actions of the CORT are rooted in the basal levels of plasma CORT at the moment of challenge. In this experiment the levels of CORT in the plasma from the 5 day CORT-treated birds, were similar at the time of challenge to the 2 day CORT-treated birds. Therefore, either the period of CORT treatment or the cumulative dose has led to immunoenhancement at least for a short period of time (1-13 days). The pre-exposure to high levels of exogenous CORT caused down-regulation of receptors of corticosteroid-binding globulin (CBG) or affinity (Armario et al 1994; Fleshner et al 1995). CBG is a plasma carrier protein important for transporting CORT in its biologically inactive form in which state it can not bind with target receptors (Pardridge 1981). A reduction in CBG reactivity would be expected to produce a measurable increase in unbound or free biologically active CORT (Mendel 1989). Free CORT was not measured in these experiments. Spencer and McEwen (1990), showed that frequent exposure to a chronic stress can decrease the neuroendocrine response, in contrast to these findings, but this may reflect the longer period of their study compared to the present research.

These findings emphasize the dynamic nature of CORT production following the imposition of stressors, which may, or may not, cause measurable changes in serum CORT levels. Hence 'one off' CORT assays may not accurately determine welfare status of poultry. Short term peaks of exogenous CORT may be detrimental and more sustained elevated levels beneficial to aspects of the immune response. Over all, these beneficial effects are short term and of uncertain value homeostatically, except for example where they may interfere with the success of a vaccination program.

The studies described in this thesis have attempted to clarify the effects of stress on the immune system of the broiler chickens, but future experiments should be designed in order to broaden the results found in this study. Several important issues remain to be addressed. The first study described in this thesis (Chapter 3) determined the prevalence of IBV and whether particular management or environmental factors weres related to its presence. If the wish of the chicken broiler industry is to produce 'free range' chickens, additional epidemiological studies should be pursued with careful choice of controls. Other risk factors, such as day length (number of hours of light per day) should be investigated as part of a production system. In the short days and long nights of winter, more melatonin is secreted than in the longer days of summer (Lam et al 2004). Several studies have demonstrated that melatonin markedly enhances macrophage activity, elevates antibody response, increases cytokine production from macrophages and T helper 1 lymphocytes (e.g., IFNγ) and produces reproductive and behaviour changes (Nelson and Demas 1997; Liu et al 2001).

In the second experiment described in this thesis (Chapter 4), it was found that the birds housed at high temperature developed a lower humoral immune response to IBV but there was no link with corticosterone levels. It would be interesting to measure the effect of high temperature on other homeostatic hormones such as the catecholamines. Certain studies have reported that elevated levels of norepinephrine, and epinephrine can induce

immunosuppression (Pedersen and Hoffman-Goetz, 2000). The use of a non-selective β-adrenergic antagonist (β blocker) would help to determine if the immunomodulatory effects (depression or enhancement) of heat stress are mediated by the activation of β-adrenergic receptors via catecholamines. Certain immune parameters were not examinated in these studies, for example local antbody (IgA) production and alterations to innate immune mechanisms, such as ά and β IFN. Some studies on the levels of T-helper 2 cytokines (e.g., IL6, IL8, IL2) could be made in order to clarify if there is any alteration in the release of those cytokines as a result of heat stress, as has been reported in rats (Wang et al 2001). Also, the phagocytic activity of blood leukocytes should be assessed, since it has been reported that high temperature reduces phagocytic activity of blood leukocytes in birds (Kadymov and Aleskerov 1988) and CORT has been reported to enhance it (Lyte et al 1990).

In conclusion, the effects of stress on these immune parameters are complex, and their relationship to the intensity or duration of the stressor is not clear, as reported by Pruett (2001). The experiments described in Chapters 4, 5 and 6 indicate that stress and the administration of CORT can exert immunoenhancing and immunosuppressive effects under differing conditions. It has been suggested that a stress-induced suppression of immune function may be evolutionarily adaptive because immunosuppression may conserve the energy that is required to deal with the immediate demands imposed by a stressor. However, immunosuppression does not necessarily conserve energy and some of the proposed mechanisms for immunosuppression, such as apoptosis of leukocytes, are likely to expend energy (Dhabhar 2002). Moreover, the immune system may often be critically needed for providing an immediate response to the actions of the stress-inducing agent (e.g., wounding by a predator). Furthermore, the time course for many proposed mechanisms for stress-induced immunosuppression, such as inhibition of prostaglandin synthesis, cytokine

production, or leukocyte proliferation (Schleimer et al 1989) is significantly longer than that seen during acute stress (Dhabhar 2002).

Environmental stressors such as low or high temperature do affect the physiology of the fast growing broiler. The adjustments that birds have to make to maintain homeostasis change the course of common infectious diseases, such as IB, that under optimal conditions commonly present in the New Zealand poultry industry, manifests itself as a mild disease.

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Appendix I

QUESTIONAIRE: INFECTIOUS BRONCHITIS

(DELETE INCORRECT ANSWER OR ADD THE CORRECT ANSWER)

ACCESSION NUMBER (lab use)………

1. **FARM NAME…………………………….SHED NUMBER (or name)………**
2. **NAME OF FRAM MANAGER……………………**
3. **DATE……………………**
4. **LOCATION ADDRESS……………………………………………PHONE………**
5. **NUMBER OF SHEDS ON FARM…………**
6. **OPEN-SIDE OR CONTROLLED ENVIRONMENTAL SHED**
7. **ALL-IN, ALL-OUT SYSTEM? YES/NO**
8. **DENSITY OF BIRDS IN THE SHED…………..(BIRDS PER SQUARE METRE)**
9. **9 BREED………………..**
10. **AGE…………**
11. **TYPE OF OPERATION (FLOOR, CAGE, FREE RANGE ETC)……………….**
12. **FEEDING PROGRAMME (RESTRICTED OR OPEN ACCESS) IF IT IS RESTRICTED EXPLAIN HOW**
13. **LEVEL OF PROTEIN IN FEED (%)……………**
14. **FEED PRESENTATION (PELLETS, CRUMBLES, ETC)**
15. **FEED CONVERSION RATIO (CURRENTLY)…………………….**
16. **WATER ACCESS (RESTRICTED OR OPEN ACCESS)………………………**
17. **WATER CONSUMPTION (PER 1000 BIRDS)………………………**

18. MEAN SHED TEMPERATURE (DAY TIME)………….(NIGHT TIME)………..

19. VENTILATION PROGRAM IN WINTER

20. WETNESS OF LITTER: Pick up a handful of litter and squeeze it tightly. When you open your hand: (A) a cohesive ball was formed or (B) crevices are formed in the compressed material or (C) the litter fell away in a pile **(DELETE THE INCORRECT ONE)**

21. TYPE OF LITTER (DESCRIBE TYPE)…………..NEW OR USED

22. FLOOR (SOIL OR CONCRETE)………………………….

23. PROGRAMME OF LIGHTING …………………………..

24. IS THE SHED EMPTIED, CLEANED, DISINFECTED, AND READIED BETWEEN BATCHES OF CHICKS (YES/NO)………….. IF YES TO PREVIOUS QUESTION, WHAT IS THE TIME PERIOD FOLLOWING THIS PROCEDURE TILL RE-STOCKING (IN DAYS)…2 weeks………..

25. VACCINATION PROGRAMME (I.B OR OTHERS)

26. DATE ILLNESS FIRST SEEN………………..

27. SIGNS OF SICKNESS…………..

28. NUMBER OF DEAD BIRDS PER DAY……………….

29. DISTANCE BETWEEN THIS SHED AND ITS CLOSEST NEIGHBOURING SHED…………..

30. HYGIENE MEASUES USED BY PERSONNEL WHEN MOVING TO AND FROM OTHER SHEDS…………………….

Appendix II

Mean of certain ambient parameters

Farm	Temp C°	Oxygen %	Ammonia ppm	Carbon dioxide %	Relative humidity %	Litter humidity (1-4)	RT-PCR
1 Case	21	20.1	20	0	89	2	negative
1 Control	21	20.3	20	0.2	97	3	negative
2 Case	21	20.3	30	0.5	70	3.5	trachea +
2 Control	20	20.5	20	0.2	70	3.5	negative
3 Case	20	20.1	20	0.7	92	3.5	negative
3 Control	20	20	20	0.8	92	3.5	negative
4 Case	22.5	20.9	18	0.1	75	1	negative
4 Control	22.5	20.5	16	0.1	65.5	1	negative
5 Case	21	20.3	15	0.1	60	3.5	cloaca +
5 Control	21.5	20.5	15	0.2	58	2	negative
6 Case	29	20.1	35	0.64	90	2	cloaca +
6 Control	28.9	20.4	5	0.48	77	2	negative
7 Case	24	20.3	7	0.15	60	3.5	tra-cloa+
7 Control	22	20.9	7	0.13	58	3	negative
8 Case	28	20.6	40	0.4	93	3	negative
8 Control	27.8	20.5	50	0.41	94	3	negative
9 Case	20	20.4	18	0.2	50	3.5	tra-cloa+
9 Control	20	20.5	18	0.2	53	3.5	tra-cloa+
10 Case	20	20.6	5	0.3	88	1	negative
10 Control	20	20.4	7	0.4	75	1	negative

Required sample size for different flock sizes, prevalences and test sensitivities to obtain a 95% certainty to detect an infection (Canon and Roe (1982)

Flock size	Expected test sensitivity	Prevalence % 50-100	Prevalence % 25	Prevalence % 10
10000	100	2	11	29
	50	4	22	58
	25	8	44	116
100000	100	2	11	29
	50	4	22	58
	25	8	44	116

Appendix III

Number of animals with specific clinical signs scores 0-3

Clinical signs birds high temperature	day 1	day 2	day 3	day 4	day 5	day 6
no clinical signs (0)	20	17	15	12	12	8
lacrimation, slight heat movement sneezing, coughing (1)		3	4	8	4	6
lacrimation, tracheal rales, nasal exudates, depression (2)			1		4	
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds high temperature	day 8	day 9	day 10	day 11	day 12	day 13
no clinical signs (0)	9	8	6	8	10	7
lacrimation, slight heat movement sneezing, coughing (1)	4	6	6	6	4	1
lacrimation, tracheal rales, nasal exudates, depression (2)	1		2			
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds high temperature	day 15	day 16	day 17	day 18	day 19	day 20
no clinical signs (0)	8	8	8	8	8	8
lacrimation, slight heat movement sneezing, coughing (1)	0	0	0	0	0	0
lacrimation, tracheal rales, nasal exudates, depression (2)						
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds low temperature	day 1	day 2	day 3	day 4	day 5	day 6
no clinical signs (0)	20	17	7	2	1	1
lacrimation, slight heat movement sneezing, coughing (1)		3	6	8	9	8
lacrimation, tracheal rales, nasal exudates, depression (2)			7	10	4	5
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds low temperature	day 8	day 9	day 10	day 11	day 12	day 13
no clinical signs (0)	2	3	3	3	4	5
lacrimation, slight heat movement sneezing, coughing (1)	8	9	9	9	9	0
lacrimation, tracheal rales, nasal exudates, depression (2)	4	2	2	2	1	3
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds low temperature	day 15	day 16	day 17	day 18	day 19	day 20
no clinical signs (0)	6	6	6	7	7	7
lacrimation, slight heat movement sneezing, coughing (1)	0	0	1	1	1	1
lacrimation, tracheal rales, nasal exudates, depression (2)	2	2	1			
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds moderate temperature	day 1	day 2	day 3	day 4	day 5	day 6
no clinical signs (0)	20	20	18	14	13	8
lacrimation, slight heat movement sneezing, coughing (1)			2	6	5	4
lacrimation, tracheal rales, nasal exudates, depression (2)					2	2
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds moderate temperature	day 8	day 9	day 10	day 11	day 12	day 13
no clinical signs (0)	10	9	10	10	10	8
lacrimation, slight heat movement sneezing, coughing (1)	3	4	4	4	4	0
lacrimation, tracheal rales, nasal exudates, depression (2)	1	1				
same as 2 but stronger, plus swollen heads (3)						

Clinical signs birds moderate temperature	day 15	day 16	day 17	day 18	day 19	day 20
no clinical signs (0)	8	8	8	8	8	8
lacrimation, slight heat movement sneezing, coughing (1)	0	0	0	0	0	0
lacrimation, tracheal rales, nasal exudates, depression (2)						
same as 2 but stronger, plus swollen heads (3)						

Clinical score of the three treatment groups over time

	High	Moderate	Low
Day 1	0	0	0
Day 2	0.15	0	0.15
Day 3	0.3	0.1	1
Day 4	0.4	0.3	1.4
Day 5	0.6	0.45	1.2
Day 6	0.4	0.4	1.28
Day 7	0.42	0.42	1.21
Day 8	0.42	0.35	1.1
Day 9	0.42	0.42	0.9
Day 10	0.71	0.28	0.9
Day 11	0.42	0.28	0.9
Day 12	0.28	0.28	0.78
Day 13	0.12	0	0.75
Day 14	0.12	0	0.5
Day 15	0	0	0.5
Day 16	0	0	0.5
Day 17	0	0	0.37
Day 18	0	0	0.12
Day 19	0	0	0.12
Day 20	0	0	0.12

Appendix IV: Number of animals with specific lesion scores (0-3)

LESIONS

Air sac	High Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	3	5	6
scored 1	3	1	
scored 2			
scored 3			

Heart	High Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	6	6	6
scored 1			
scored 2			
scored 3			

Liver	High Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	6	1	4
scored 1	0		2
scored 2		5	
scored 3			

Kidney	High Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	6	6	6
scored 1			
scored 2			
scored 3			

Trachea	High Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	4	5	6
scored 1	2	1	
scored 2			
scored 3			

Air sac	Moderate Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	5	6	6
scored 1	1		
scored 2			
scored 3			

Heart	Moderate Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	6	6	6
scored 1			
scored 2			
scored 3			

Liver	Moderate Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	6	6	6
scored 1			
scored 2			
scored 3			

Kidney	Moderate Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	6	6	6
scored 1			
scored 2			
scored 3			

Trachea	Moderate Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	5	6	6
scored 1	1		
scored 2			

Air sac	Low Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0		6	6
scored 1	6		

Heart	Low Day 6p.i	 Day 13 p.i	 Day 20 p.i
scored 0	3	1	5
scored 1	2	2	

scored 2			
scored 3			

Liver	Low Day 6p.i	Day 13 p.i	Day 20 p.i
scored 0	4	3	3
scored 1	2	3	3
scored 2			
scored 3			

Trachea	Low Day 6p.i	Day 13 p.i	Day 20 p.i
scored 0	2	5	6
scored 1	3	1	
scored 2	1		
scored 3			

scored 2			
scored 3	1	3	1

Kidney	Low Day 6p.i	Day 13 p.i	Day 20 p.i
scored 0	4	4	6
scored 1	2	2	
scored 2			
scored 3			

Composite lesion score (mean) in each treatment group

Lesions found in birds 6 days after challenge

	Tracheal lesions	Air sac lesions	Heart lesions	Liver lesions	Kidney lesions
High	0.33	0.5	0	0	0
Moderate	0.16	0.16	0	0	0
Low	0.83	1	0.83	0.33	0.33

Lesions found in birds 13 days post challenge

	Tracheal lesions	Air sac lesions	Heart lesions	Liver lesions	Kidney lesions
High	0.16	0.33	0	1.66	0
Moderate	0	0	0	0	0
Low	0	0	1.8	0.5	0.33

Lesions found in birds 20 days post challenge

	Tracheal lesions	Air sac lesions	Heart lesions	Liver lesions	Kidney lesions
High	0	0	0	0.33	0
Moderate	0	0	0	0	0
Low	0	0	0.5	0.5	0

Appendix V

Spleen and body weights (% = spleen/body weights)

	Body weight grams	Spleen weight grams	%
Bird 1 (control)	2089	1.7	0.08
Bird 2 (control)	2028	1.5	0.07
Bird 3 (control)	1705	1.4	0.08
Bird 4 (control)	1962	1.8	0.09
Bird 5 (control)	1827	2.2	0.12
Bird 6 (control)	2054	1.6	0.07
Bird 1 (IBV SRBC)	1709	1.5	0.08
Bird 2 (IBV SRBC)	1802	1.7	0.09
Bird 3 (IBV SRBC)	1819	1.6	0.08
Bird 4 (IBV SRBC)	1715	1.5	0.08
Bird 5 (IBV SRBC)	1867	1.8	0.09
Bird 6 (IBV SRBC)	1717	1.5	0.08
Bird 1 (IBV SRBC Cort)	1064	0.3	0.028
Bird 2 (IBV SRBC Cort)	1066	0.4	0.037
Bird 3 (IBV SRBC Cort)	1458	1	0.068
Bird 4 (IBV SRBC Cort)	1256	0.5	0.039
Bird 5 (IBV SRBC Cort)	1496	1.2	0.08
Bird 6 (IBV SRBC Cort)	1302	0.9	0.069

Appendix VI

Solutions for INF γ test

SOLUTION	FORMULATION
Coating Buffer	8.0 g NaCl 1.42 g $Na_2HPO_42H_2O$ 0.2 g KH_2PO_4 0.2 g KCl q.s. to 1 litre with distilled H_2O, pH 7.4
Blocking Solution	8.0 g NaCl 1.42 g $Na_2HPO_42H_2O$ 0.2 g KH_2PO_4 0.2 g KCL 5.0 g bovine serum albumin (fraction V) q.s. to 1 litre with distilled H_2O, pH 7.4
Wash Buffer	9.0 g NaCl 1 mL Tween 20 q.s. to 1 litre with distilled H_2O, pH 7.4
Stop Solution	1.8 NH_2SO_4